INTERACTION OF
ARGININE-RICH HISTONE AND DEOXYRIBONUCLEIC ACID

by

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ABSTRACT

INTERACTION OF ARGININE-RICH HISTONE AND DNA

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Thermal denaturation and circular dichroism were used to study the interaction of Arg-rich histones (H3 + H4), polyarginine, poly(Arg^{87}, Orn^{13}), and protamine with DNA.

Complexes of DNA with polyarginine, Poly(Arg^{87},Orn^{13}), and protamine prepared by direct mixing method showed biphasic melting with a melting band at a melting temperature, T_m, corresponding to free base pairs and another melting band or bands at T_m' corresponding to protein-bound DNA base pairs. Protaine-DNA complexes have one T_m' melting band, while polyarginine and poly(Arg^{87}, Orn^{13})-DNA complexes have two T_m' bands ($T_{m,I}'$ and $T_{m,II}'$). In the latter complexes the melting temperatures and the ratio of melting areas of these two T_m' bands are a function of the GC content in the DNA used. Protein-bound DNA base pairs are almost completely shielded electrostatically. The β values (amino acid residues per nucleotide in the protein-bound regions) obtained for these complexes are 0.72 for polyarginine-DNA complexes, 1.05 for poly(Arg^{87},Orn^{13})-DNA complexes, and 1.38 for protamine-DNA complexes. The CD spectra of these complexes show reduced amplitude and a red-shift for the DNA CD near 275 nm.

Calf thymus histone H3 monomer, dimer, oligomers and duck erythrocyte histone H3 monomer and dimer were purified by a modified procedure of Sanders and McCarty (1972). Analysis of calf thymus histone H3 monomer CD spectra indicate that histone H3 has more secondary structures in EDTA buffer than in water or acetic acid and is essentially a random coil in urea. CD spectra also indicated that histone H3 dimer has more secondary structure than monomer in water or in EDTA buffer.

The method of direct mixing yieldes histone H3-DNA complexes with well defined melting curves. For calf thymus histone H3 monomer-DNA complexes, a T_m' melting band at 90^o and a β value of 6.7 amino acid residues per nucleotide were obtained. These complexes show a reduction in amplitude and a slight red-shift in the DNA CD band near 275 nm and a big negative CD band near 220 nm contributed by the bound histones. The change in CD amplitude near 220 nm for calf thymus histone H3 monomer from a free state in EDTA solution, $\Delta\varepsilon_f^H$ (EDTA), to a bound state, $\Delta\varepsilon_b^H$, is small. Similar thermal denaturation and CD results with minor variations were obtained for directly mixed DNA complexes using calf thymus histone H4, H3 dimer, oligomers, and duck histone H3 monomer and dimer.

Gradient dialysis with or without urea were also used to prepare histone-DNA complexes. These two methods yield histone H3-DNA complexes with less defined melting curves. The presence of both histone H3 and H4 during complex formation with DNA in these two procedures yields complexes that are considerably more soluble with better melting curves. These results indicate the importance of histone H3 and H4 interaction in complexing with DNA.

Complexes DNA with histone (H3 + H4) tetramer, prepared by the method of D'Anna and Isenberg (1974c), were also studied. These complexes have two melting bands at 65° and 90° corresponding to histone-bound DNA and have a β value of 2.8 amino acid residues per nucleotide. The CD spectra of these complexes show a reduction in amplitude and a slight red-shift for the DNA CD band near 275 nm. These complexes also have a strong negative CD band near 220 nm corresponding to the bound histones. These thermal denaturation and CD results of tetramer-DNA complexes show a large degree of similarity to those of chromatin.

Histone (H3 dimer + H4) tetramer-DNA complexes have thermal denaturation and CD properties similar to those obtained for histone (H3 + H4) tetramer-DNA complexes. These results suggest that the two histone H3 molecules possibly form a parallel dimer with or without disulfide bond and that this dimer is a basic subunit for the tetramer, which seems to be a fundamental subunit in chromatin.

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CHAPTER I

INTRODUCTION

The wonder of cell nuclei lies in the packaging of and the selective information retrieval from the DNA molecules. In an interphase nuclei, DNA molecules are found in chromatin as a complex macromolecular association with proteins and RNA. Proteins in chromatin may be divided into two groups, the basic group called histones and the non-basic group called non-histone proteins. Histones play a major role in the maintenance of structures in chromatin and possibly in template restriction, while the non-histone proteins are important in genetic regulation (Elgin et al., 1971). However, the precise roles of these proteins are still not clear.

There are five main types of histone found in eukaryotes. Some of their physical and chemical properties are summarized in Table I. The avian, frog and fish erythrocytes also contain a specialized histone called histone H5 (V or f2c), this histone is not found in most other cell types (Hnilica, 1972). All histones have low molecular weights (11,000-21,000). Histones are basic and can easily be extracted from chromatin by acids. All histones studied so far do not contain

TABLE I Properties of Histone Fractions[a]

Nomenclature	Molecular Weight	Number of Amino Acid Residues	NH_2-terminal	Lys/Arg	Class
H1 (I or f1)	21,000	212-216	Ac-Ser	20	Lys-rich
H2B (IIb2 or f2b)	13,774	125	Pro	2.5	Slightly Lys-rich
H2A (IIb1 or f2a2)	14,000	131	Ac-Ser	1.0-1.2	Slightly Lys-rich
H3 (III or f3)	15,324	135	Ala	0.72	Arg-rich
H4 (IV or f2a1)	11,282	102	Ac-Ser	0.79	Arg-rich

(a) The data in this table are those of calf thymus (DeLange and Smith, 1971; Hnilica, 1972). However, other species histones are very similar.

tryptophan and cysteine residues except histone H3 (III or f3) which may contain one or two cysteine residue depending on its source. It is noteworthy that three of the histones contain acetyl-Ser as their N-terminal residue (DeLange and Smith, 1971). Histones are usually classified by their lysine and arginine contents. Histone H1 (I or f1) and histone H5 (V or f2c) are rich in lysine and are called the very Lys-rich histones. Histones H2A (IIb1 or f2a2) and H2B (IIb2 or f2b) are classified as slightly Lys-rich histones, while histone H3 and H4 are the Arg-rich histones. There are approximately equal weights of histone and DNA in chromatin. This is true from a variety of tissues and species (Elgin and Bonner, 1970). There is roughly an equal molar amount of each histone fraction except histone H1 which is usually only half as much as the others (Panyim and Chalkley, 1969).

The complete primary sequences of the five histones have been determined. Comparative studies of histone sequences from different sources have revealed a highly conserved nature of the primary sequences. Histone H4 is by far the most conserved histone in which only two of the 102 amino acid residues are different when isolated from two highly diverged sources, that of calf thymus and pea seedlings (DeLange et al., 1969). The two amino acid changes are also of highly conservative nature, i.e., Val is changed to Ile and Lys to Arg. Similar studies with histone H3 isolated from pea and calf thymus indicated that these histones with 135 residues differ from each other only in four places (Patthy et al., 1973). The calf thymus sequence differs from those of shark, carp and chicken by only one residue. Ser 96 is changed to Cys in calf thymus. The slightly Lys-rich histones (H2A and H2B) are less conserved than the Arg-rich histones (H3 and H4) (DeLange and Smith, 1971; Rall and Cole,

1971) and the Lys-rich histone H1 varies to a great extent among species.

The highly conserved nature of primary sequences in Arg-rich histones suggest the importance of their entire sequences in their interaction within the chromatin. It further suggests that histone H3 and H4 might have close interaction with the sugar-phosphate helical backbone of DNA since the latter is also unaltered in the evolutionary changes.

Histones, once synthesized and interacted with DNA in the chromatin, have very long half-life (Hancock, 1969). This suggests the important and unique nature of histones in the maintenance of chromatin structure. However, extensive side chain modification has been found in histones, some of which are stable while the others are metabolically unstable and turnover rapidly according to cellular changes (DeLange and Smith, 1971). A summary of histone modifications is shown in Table II. The Arg-rich histones have acetylated and methylated Lys residues. The acetylation reaction seems to correlate with control of RNA synthesis (Wilhelm and McCarty, 1970), while methylation reaction seems to be related with mitosis (Tidwell et al., 1968).

As mentioned previously, histone H3 is the only cysteine containing histone. Higher animals contained two cysteines while the others contain only one (Panyim et al., 1971). Because of the presence of cysteine residues, oxidation of two -SH groups into a disulfide can form dimeric and in the case of higher animals, polymeric forms of histone H3. Several laboratories (Cross and Ord, 1970; Sadgopal and Bonner, 1970) have reported that the ratio -SH/[(-SH) + (-S-S-)] is high in interphase chromosomal proteins with high transcriptional activities, and that their ratio is low during metaphase, when chromatin is in an aggregated form and their transcriptional activities are low. Since chromatin appears to be more

TABLE II

Side Chain Modification of Histone Fractions

Modifications	Histone fractions modified	Metabolic stability	Reference
Acetyl-Ser (N-terminal)	H1, H2A, H4	Stable	(a)
ϵ-N-acetyl-Lys	H3, H4 (H2A, H2B)	Turn-over	(b)
ϵ-N-methyl-Lys	H3, H4	Slow turn-over	(c)
O-phospho-Ser	H1 (H2A, H2B, H3, H4)	Turn-over	(d)
O-phospho-Thr	H1	-	(d)
ω-methyl-Arg	(g)	(g)	(e)
ADP-Ribosyl-	(g)	(g)	(f)

(a) DeLange and Smith, 1971
(b) Wilhelm and McCarty, 1970
(c) Paik and Kim, 1967
(d) Stevely and Stocken, 1966
(e) Paik and Kim, 1969
(f) Nishizuka et al., 1968
(g) Unknown

condensed in metaphase than in interphase, it is conceivable that formation of intermolecular disulfide linkage between histone H3 can bring different parts of chromatin closely together and could be an important structural element in condensed chromatin.

Comparative studies of histone fractions from different sources have pointed out the conservation of their primary sequences. The primary sequences of different histone fractions from the same source, calf thymus for instance, also show some striking similarity in residue distribution. Distribution of basic residues along histone molecules are found to be universally uneven that one half of each histone molecule contains more basic residues (N-terminal half in histone H2A, H2B, H3 and H4 and C-terminal half in histone H1) and the other half more hydrophobic residues (LeLange et al., 1973).

The existence of a more basic and a less basic half in a histone molecule has led to the proposal that the two ends of histone molecules electrostatically stabilized DNA differently. The DNA regions stabilized by the more basic half melt at a higher temperature than those stabilized by the less basic half (Li and Bonner, 1971). This proposal was further supported by the use of two halves of histone H2B molecules. H2B was cleaved by CNBr at the Met residues (59 and 62), which are located in the middle of the molecule. DNA complexed with the N-terminal fragment melts at a temperature of 70°C while that with the C-terminal fragment melts at 57°C. This model can also explain the two melting bands (81°C and 66°C) in native chromatin.

Currently a subunit model of chromatin has become very popular (Kornberg and Thomas, 1974; Van Holde et al., 1974; Baldwin et al., 1975; Olins and Olins, 1974; Li, 1975). The proposals suggested that chromatin

consists of a linear array of nearly identical subunits. The proposal derives from the findings of nucleases resistant fractions of chromatin, about 50% in calf thymus, containing particles with about 200 base-pairs of double stranded DNA and the normal complement of histones (minus H1) (Van Holde et al., 1974). Electron microscopic studies of swollen, burst nuclei also show chromatin with a "string of beads" structure (Olins and Olins, 1974).

Recently results from histone-histone interaction further supported this subunit model of chromatin. It was found that specific histone fractions form stoichiometric soluble complexes with high affinity (D'Anna and Isenberg, 1974b). The arginine-rich histones, H3 and H4, have been found to form a very tight tetrameric structure [2 (H3) + 2 (H4)] (D'Anna and Isenberg, 1974c). Chemical cross-linking study on chromatin also revealed a close interaction of the two histones (Hyde and Walker, 1975). Extraction of histones from chromatin by non-denaturating condition results in a simultaneous removal of histones H3 and H4 as a tetrameric complex (Kornberg and Thomas, 1974; Roak et al., 1974). Other histone-histone interactions have also been shown to exist between H2A and H2B (D'Anna and Isenberg, 1974b; Kelly, 1973; Kornberg and Thomas, 1974; Hyde and Walker, 1975) and between H2A-(or H2B) and H4 (D'Anna and Isenberg, 1974b; Martinson and McCarthy, 1975). Histone H1 was found not to be involved in any complex formation with other histones. A summary of complex formation among histones is shown in Table III. Since all histones (except H1) contain a basic N-terminal and a hydrophobic C-terminal portion, histone-histone interaction is probably stabilized through hydrophobic interaction between histones at their C-terminal regions.

TABLE III

Complex Formation Among Histones

Histone complexes	Number of subunit	K_a or F	Method of identification	References
H3 + H4	tetramer	$K_a = 0.7 \times 10^{21}\ M^{-1}$	Complex formation in phosphate buffer, M.W. determination	D'Anna, Jr., and Isenberg (1974c)
	tetramer	-	Chemical cross-linking	Kornberg and Thomas (1974)
	Dimer and tetramer	F = -3.6 -Kcal/mole	M.W. determination	Roaks et al. (1974)
	dimer to polymer	-	Chemical cross-linking on chromatin	Hyde and Walker (1975)
H2A + H2B	dimer to polymer	-	Chemical cross-linking	Kornberg and Thomas (1974)
	dimer	-	Complex formation M.W. determination	Kelly (1973)
	dimer	$K_a = 10^6\ M^{-1}$	Fluorescence and CD	D'Anna, Jr., and Isenberg (1974b)
	dimer to polymer	-	Chemical cross-linking on chromatin	Hyde and Walker (1975)
H2B + H4	dimer	$K_a = 10^6\ M^{-1}$	Fluorescence and CD	D'Anna, Jr., and Isenberg (1974b)
	dimer	-	Chemical cross-linking on chromatin	Martinson and McCarthy (1975)

A dynamic equilibrium model of chromatin has recently been proposed by Dr. H.J. Li (1975). The model suggests that the observed chromatin structures are affected by external factors such as ionic strength, type of ions, pH etc. and can exist as either a "string of beads" or a super coiled structures.

In view of the importance of histone-DNA and histone-histone interactions, detailed studies on interaction of DNA with histone H3 monomer, dimer and oligomer and with histone (H3 + H4) tetramer have been studied, using the methods of thermal denaturation and circular dichroism. In addition, since histone H3 and H4 are arginine-rich histones, in the course of this thesis research, interaction between DNA and polyarginine, and between DNA and protamine, containing 67 mole percents of arginine, has also been extensively investigated.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Calf thymus DNA was purchased from Sigma Chemical Co. Protamine was purchased from Sigma Chemical Co. and was dissolved and dialyzed against 2.5×10^{-4}M EDTA, pH8.0. The concentration of protamine stock solution in EDTA was determined either by the Micro-Biuret Method (Itzhaki and Gill, 1964) using histone as the standard, or by the ninhydrin method (Spies, 1957) using arginine chloride as the standard. Poly (L-arginine) sulfate (mol wt 14,000) was purchased from Miles Laboratories. The purity was confirmed by amino acid analysis as 99% in arginine. Poly (L-arginine) hydrochloride (mol wt 65,000) was purchased from Sigma Chemical Co. Amino acid analysis showed that this sample happened to contain 13% ornithine. This sample was used as poly (Arg^{87}, Orn^{13}). Stock solutions of polyarginine and poly (Arg^{87}, Orn^{13}) were made in and dialyzed against EDTA buffer before use. Their concentrations were determined both by ninhydrin method using arginine as the standard (Spies, 1957) and amino acid analysis, using modified automated amino acid analyzer (Liao et al., 1973).

Isolation and purification of histone H3 from calf thymus and duck erytrocytes will be reported in chapter IV. Crude histone H3 fraction from duck erythrocytes was a gift of Dr. Lee Sanders and purified histone H4 from calf thymus was a gift of Dr. T.Y. Shih.

PURIFICATION OF DNA

100 mg of calf thymus DNA were dissolved in 50 ml of 0.05 M Tris-HCl, pH 8.0, 0.05 M NaCl, 1 mM EDTA overnight with a slow stirring at 4°C. An equal volume of buffer saturated-phenol was then added and stirred with the DNA solution for 5 minutes. Aqueous and phenol phases were then separated by a 12,000 x g centrifugation for 10 minutes. The aqueous phase was removed and extracted again with an equal volume of buffer-saturated phenol. This step was repeated until no detectable denatured proteins were found at the water-phenol interphase. Phenol was then removed from the DNA solution by extensive dialysis against NaCl-Tris buffer. DNA solutions were further dialyzed against 2.5×10^{-4} M EDTA before use. The molar extinction coefficient of 6500 $M^{-1}cm^{-1}$ at 260 nm was used for DNA, DNA-polypeptide and DNA-histone complexes where M is mole/liter of nucleotide.

PREPARATION OF POLY-L-ARGININE-CHLORIDE

Poly-Arg-sulfate was converted into the chloride form to render it water soluble. Concentrated HCl was added to a suspension of poly-Arg-sulfate in water until pH 3.45. The mixture was then heated to 60°C in a water bath to dissolve the complex. $SO_4^{=}$ ion were then

removed by the addition of $Ba(OH)_2$. The mixture was chilled in ice and centrifuged to removed $BaSO_4$. The clear supernatent was then dialyzed extensively against 2.5×10^{-4}M EDTA, pH8.0.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF HISTONES

High resolution disc gel electrophoresis of histones was performed as described by Panyim et al., (1969a). Polyacrylamide gels were prepared by mixing the following solution: one part of 43.2% acetic acid (HOAc), 4% N, N, N', N'- tetramethylethylene-diamine; two parts of 60% acrylamide, 0.4% bis-acrylamide and five parts of 0.2% ammonium persulfate in 10 M urea. All solutions were deaerated before polymerization. Several 0.6 x 10 cm gels were polymerized for at least 60 minutes before use. Gels were preelectrophoreized in 0.9 N HOAc for one to two hours at a constant current of 2 ma/tube until the voltage reaches 120V. After preelectrophoresis, the HOAc tray buffers were changed before sample application. Samples containing 10-20 μg of pure histone fractions or 30-50 μg of crude histones were usually applied in 20-30 μl of 6M urea, 0.9N HOAc buffer. Pyronine-Y was used as a tracing dye. The gels were electrophorized at room temperature for 2-3 hours at 2 ma/tube and stained for at least one hour in 0.1% amido black, 20% ethanol, 7% HOAc. Destaining was done by shaking the stained gels at 30°C in 20% ethanol, 7% HOAc until the gels were clear. Gels were scanned by a Gilford Linear Transport devices at 600 nm.

PREPARATION OF PROTEIN-DNA AND POLYPEPTIDE-DNA COMPLEXES

A. Direct mixing method

To a DNA solution with a concentration of 1×10^{-4}M in nucleotide was added dropwise a polypeptide or protein solution of 10^{-3}M in amino acid residues. The DNA solution was constantly stirred while polypeptide or protein solution was added. Both the DNA and the polypeptide or protein solutions were prepared in 2.5×10^{-4}M EDTA, pH 8.0. The complexes were let stand for at least one hour before measurements were made.

B. Reconstitution methods

(1) NaCl gradient dialysis in the presence of urea

Reconstitution of histones to DNA was made by a continuous NaCl gradient (2.0 to 0.1M) dialysis in the presence of 5M urea-0.01 M Tris (pH 8.0) for two days. Urea was then continuously dialyzed out in the presence of 0.015 M NaCl-0.01 M Tris (pH 8.0). The complexes were finally dialyzed against 2.5×10^{-4}M EDTA (pH 8.0) for measurements.

(2) NaCl gradient dialysis in the absence of urea

Reconstitution of histones to DNA was done by a continuously decreasing gradient of NaCl (2.0-0.1M) in 0.01M Tris (pH8.0) for two days. The complexes were then extensively dialyzed against 2.5×10^{-4}M EDTA (pH 8.0).

THERMAL DENATURATION STUDY

A. Thermal denaturation measurements

Thermal denaturation of polypeptide- and protein-DNA complexes were performed at 260 nm using a Gilford Spectrophotometer model 2400-S at a constant heating rate of ca. $2/3^{\circ}$ per minute. The increased hyperchromicity at temperature T, h_{260} (T), referred to A_{260} at 25° was recorded degree by degree.

B. Analysis of thermal denaturation data:

(1) The derivatives of a melting profile were calculated according to the equation of Li and Bonner (1971)

$$\frac{dh_{260}\ (T)}{dT} \simeq \frac{h_{260}\ (T+1)-h_{260}(T-1)}{2}$$

where h_{260} (T) is the hyperchromicity at 260 nm and temperature T.

(2) Calculation of β value, the average number of amino acids per nucleotide in a complex:

(a) If the hyperchromicities of DNA base pairs in a complex are independent of protein or polypeptide binding, then the following equation can be used (Li, 1973)

$$r_k = \beta_k \frac{A_k}{A_T} \qquad (1)$$

where r_k is the total amino acids of protein k per nucleotide, A_k is the area under melting band k of protein-bound base pairs and A_T the total melting area which is equal to h_{max}. From equation (1), we can get β_k, the average number of amino acid residues per nucleotide in those DNA regions bound by protein k.

(b) If the hyperchromicity of protein-bound regions is different from that of protein-free regions, Eq. (1) has to be modified. In order to analyze the results for these kinds of complexes, the following equations can be used (Li et al.,1973). Let h_b, h_f, A_b and A_f be, respectively, the hyperchromicities (h) and the areas of melting bands (A) of base pairs bound by (h_b and A_b) and free of (h_f and A_f) protein. If A_T is the total melting area

$$A_T = A_b + A_f \qquad (2)$$

If F is the fraction of base pairs tightly bound by protein which melt at temperature corresponding to melting of A_b, (1-F) will be the fraction of base pairs free of protein. We then obtain

$$A_b = h_b F \qquad (3)$$

$$A_f = h_f (1-F) \qquad (4)$$

Before the binding is saturated, there is a linear relation between r, the input ratio of amino acid residues per nucleotide, and F.

$$r = \beta F \qquad (5)$$

where β is the average number of amino acid per nucleotide in bound regions. From Eqs. (3) and (5)

$$r = \frac{\beta}{h_b} A_b \qquad (6)$$

From Eqs. (4) and (5)

$$r = \beta\left(1-\frac{A_f}{h_f}\right) \qquad (7)$$

The linear plot of r against $\left(1-\frac{A_f}{h_f}\right)$ gives β from which we can determine h_b from Eq. (6). Since r, h_f, A_f and A_b can be determined experimentally, the parameters β and h_b can be determined from Eqs. (6) and (7).

CIRCULAR DICHROISM STUDY

A. Measurements of CD spectra

The CD spectra were taken on a Durrum-Jasco Spectropolarimeter model J-20 at room temperature. The results are reported as $\Delta\epsilon_m = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are, respectively, molar extinction co-

efficients for the left-and the right-handed circularly polarized light. The units of $\Delta\epsilon_m$ are $M^{-1}cm^{-1}$ in terms of nucleotide.

B. Analysis of CD spectra

According to Eq. (5), the fraction of base pairs bound by protein in each complex, F, can be determined from thermal denaturation data. If it is assumed that the CD of a complex can be decomposed into two components (Chang, et al., 1973), $\Delta\epsilon_f^D$ of free base pairs in B-form conformation and $\Delta\epsilon_b^D$ of bound base pairs whose conformation is to be determined, the following equations can be written

$$\Delta\epsilon_m = (1-F)\ \Delta\epsilon_f^D + F\ \Delta\epsilon_b^D \qquad (8)$$

$$\Delta\epsilon_m - \Delta\epsilon_f^D = F\ (\Delta\epsilon_b^D - \Delta\epsilon_f^D) \qquad (9)$$

Eq. (9) describes the fact that the difference CD spectrum is proportional to F which, in turn, is proportional to r, the input ratio of protein to DNA. From Eqs. (8) and (9) we can get CD of bound base pairs $\Delta\epsilon_b^D$.

CHAPTER III

STUDIES ON POLY (L-ARGININE)-, POLY(L-ARGININE^{87}, L-ORNITHINE^{13})- AND PROTAMINE-DNA COMPLEXES

It was anticipated that one of the major interactions between arginine-rich histones and DNA will be those of charge-charge interactions between the arginine residues and the other basic residues in histones with the phosphate groups in DNA. As a model for later studies on the arginine-rich histone-DNA complexes, polyarginine, which is a synthetic polymer containing only arginine residues, and poly (Arg^{87}, Orn^{13}), a random copolymer with 87% of arginine and 13% of ornithine were used. In these studies, protamine, a DNA-bound protein found in sperm nuclei containing 67% of its amino acid residues as arginine was also used. All the polypeptide- and protein-DNA complexes studied here were prepared by the slow and direct mixing method.

RESULTS

A. Titration of Polyarginine, Poly (Arg^{87}, Orn^{13}) and Protamine with DNA

Generally, when the negative charges of the DNA phosphates are neutralized with the basic amino acid residues of polypeptides, or protein, precipitation of the DNA-polypeptide or DNA-protein complexes occurs when certain input ratios of protein to DNA are reached. This

is true when polylysine is used to titrate DNA (Clark and Felsenfeld, 1971). Precipitation occurs at an equal molar ratio of lysine to phosphate. This is also true for the titration of DNA with poly (Arg^{87}, Orn^{13}) or protamine. Figures 1 and 2 show titration curves of DNA by poly (Arg^{87}, Orn^{13}), polyarginine or protamine. At low input ratios of amino acid per nucleotide (r) all the complexes are soluble and remain in the supernatant. Precipitation occurs in a narrow range of r and indicates that the binding of the basic polypeptides and protamine on DNA molecule is not cooperative. The midpoints of the precipitation curves are, respectively, 0.98 and 1.03 basic amino acid residue per nucleotide for poly (Arg^{87}, Orn^{13}) and protamine. However, charge neutralization may not be the only controlling factor in the precipitation of polyarginine-DNA complexes. The midpoint of polyarginine-DNA titration curve is 0.8 at which point charges on DNA phosphates are not fully neutralized. As shown in Figures 1 and 2 precipitation curves of the complexes depend not only on the nature of polypeptide used but also on the types of DNA used as well.

B. Thermal Denaturation Study

(1) Derivative melting profiles

Figures 3, 4 and 5 show, respectively, derivative melting curves of polyarginine-DNA, poly (Arg^{87}, Orn^{13})-DNA and protamine-DNA complexes. The melting curves showed typical biphasic melting profile with a T_m corresponding to the melting of free DNA regions and a T'_m corresponding to that of bound DNA regions. As expected, when more polypeptide or protein is bound to DNA, the melting band of free DNA regions at T_m

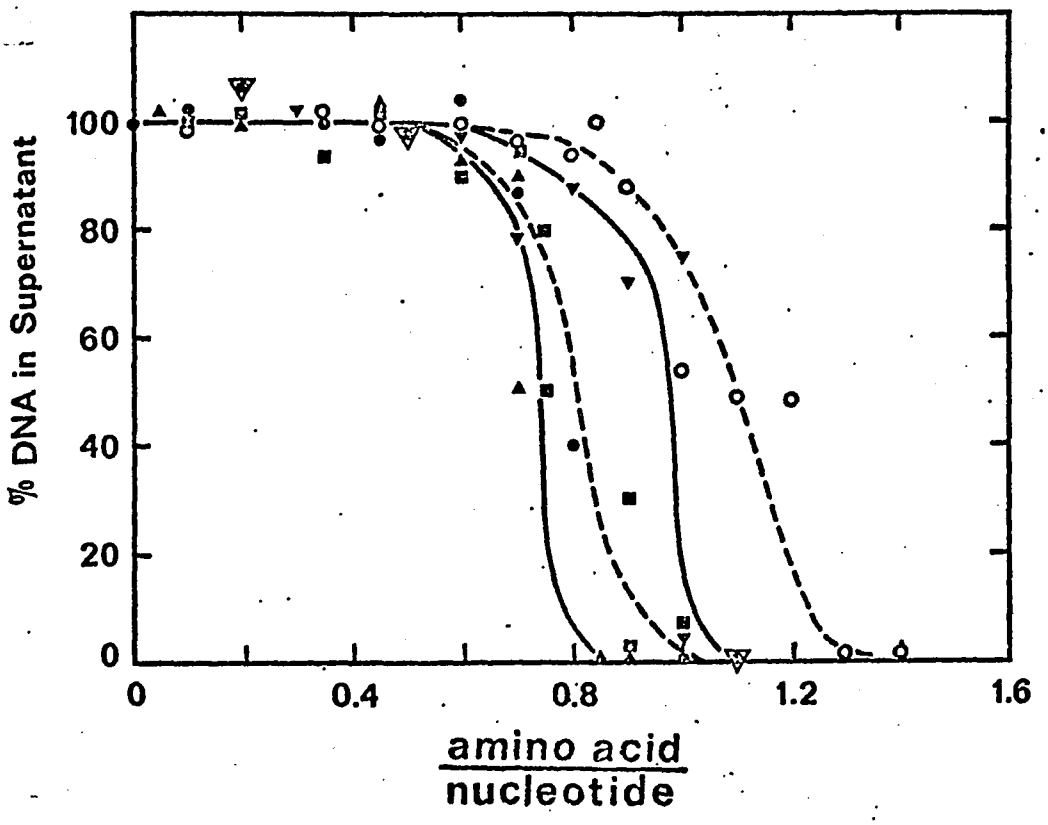


Fig. 1. Titration curves of DNA with varied GC contents by polyarginine and copoly $(arg)^{87}(orn)^{13}$. Titrated by polyarginine is DNA from Cl. perfringens (o---o), calf thymus (■---■), E. coli (▲---▲) and M. luteus (●---●). Titrated by copoly $(arg)^{87}(orn)^{13}$ is DNA from calf thymus (▼---▼).

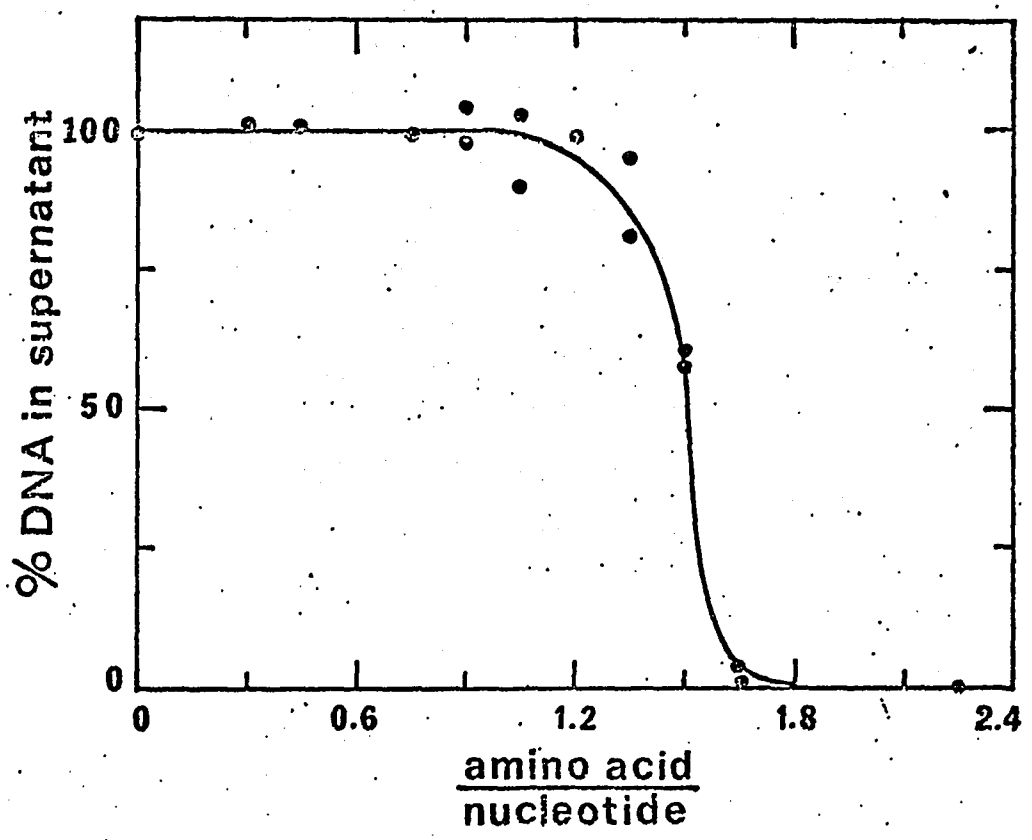


Fig. 2. Titration curve of DNA by protamine. The input ratio of protamine to DNA is reported as amino acid per nucleotide.

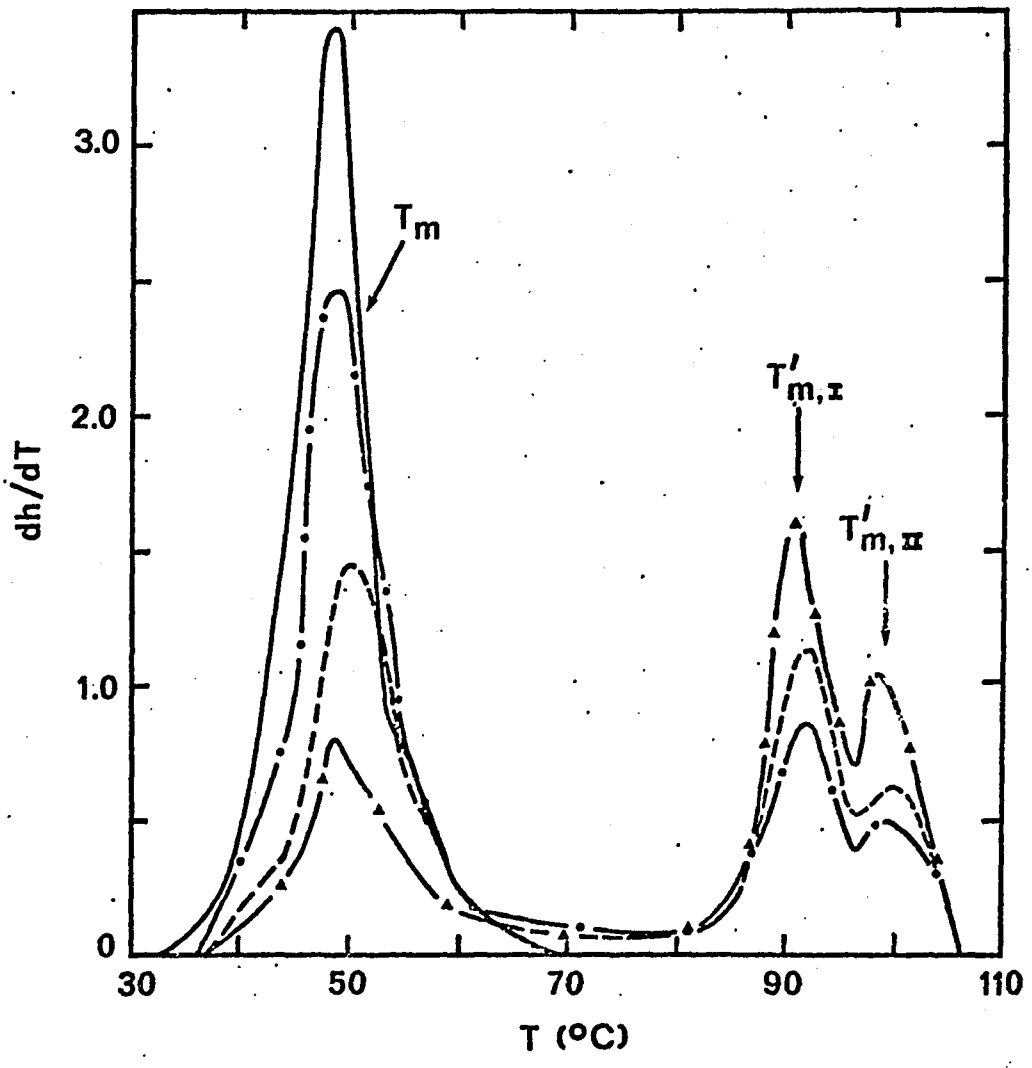


Fig. 3. Derivative melting profiles of polyarginine-calf thymus DNA complexes. The input ratio of amino acid residue per nucleotide (r) = 0 (——), 0.30 (—·—), 0.45 (----) and 0.60 (—▲—).

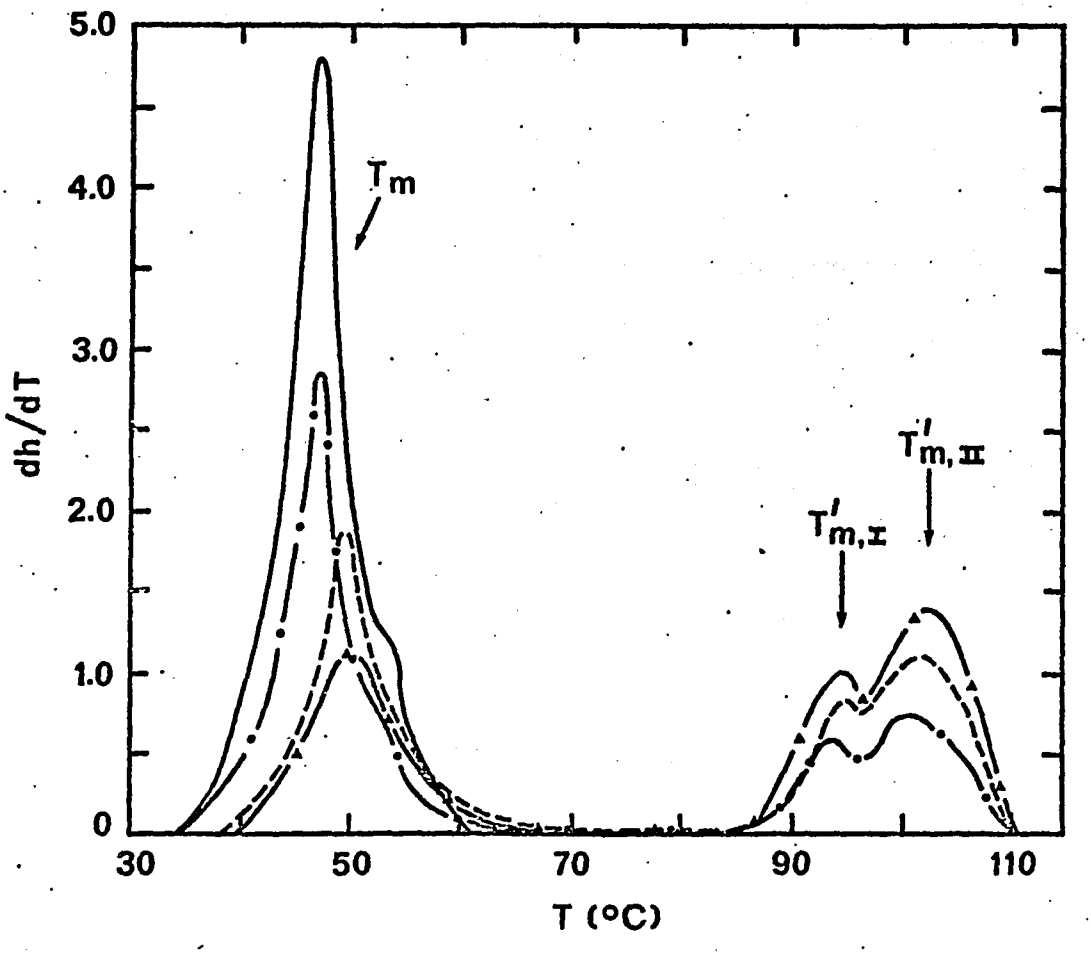


Fig. 4. Derivative melting profiles of copoly $(arg)^{87}(orn)^{13}$-calf thymus DNA complexes. r = 0 (——), 0.40 (—·—), 0.60 (----) and 0.80 (—▲—).

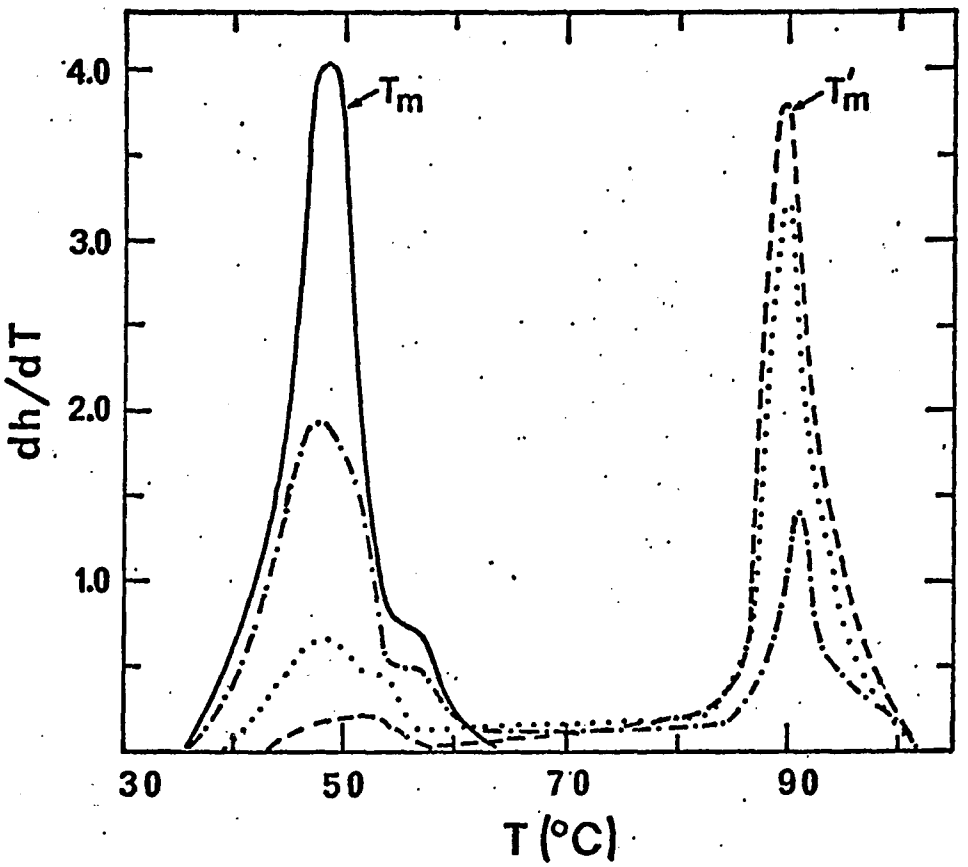


Fig. 5. Derivative melting profiles of protamine-DNA complexes. r = 0 (——), 0.3 (—·—), 0.9 (····) and 1.2 (----).

(about 50°) is decreased while that of bound regions at T_m' [about 91° for protamine; $T'_{m,I}$ about 91°, $T'_{m,II}$ about 100° for polyarginine and $T'_{m,I}$ about 94°, $T'_{m,II}$ about 102° for poly (Arg^{87}, Orn^{13})] is increased. This phenomenon is true for every basic polypeptide-DNA complexes studied. Polyarginine- and poly(Arg^{87}, Orn^{13})-DNA complexes differ from polylysine-DNA (Tsuboi et al., 1966; Li et al., 1973) or protamine-DNA complexes in that there appears two melting bands at T_m' region, $T'_{m,I}$ and $T'_{m,II}$, for the polyarginine-DNA and poly (Arg^{87}, Orn^{13})-DNA complexes while there appears only one melting band at T_m' for the others. The existence of two melting bands at T_m' region is not an artifact of EDTA buffer used for the complexing and melting experiments. Two melting bands at T_m' were also observed when the complexes were made and examined at 0.005 M cacodylate buffer, pH 7.0. The two melting bands at T_m' is not unique for the polyarginine-calf thymus DNA complex. It is also found when polyarginine is complexed with M. luteus, E. coli and Cl. perfringens DNA. It is well known that the T_m of DNA varies in a linear fashion with the G+C content (Marmur and Doty, 1962). This linear relationship between G+C content and melting temperature is also true when the T_m' of the polyarginine bound regions were used. Figure 6 shows that both $T'_{m,I}$ and $T'_{m,II}$ increase linearly with the G+C of the input DNA and that the two lines are parallel to each other while the T_m line has a different slope.

A further correlation is found between melting areas at $T'_{m,II}$ to that at $T'_{m,I}$ and the G+C content of DNA used. As indicated in Table IV this ratio decreases with the increasing G+C content of DNA. Cl. perfringens DNA containing 31% G+C has about twice as much melting area at $T'_{m,I}$ than at $T'_{m,II}$ while M. luteus containing 70% G+C has ten times

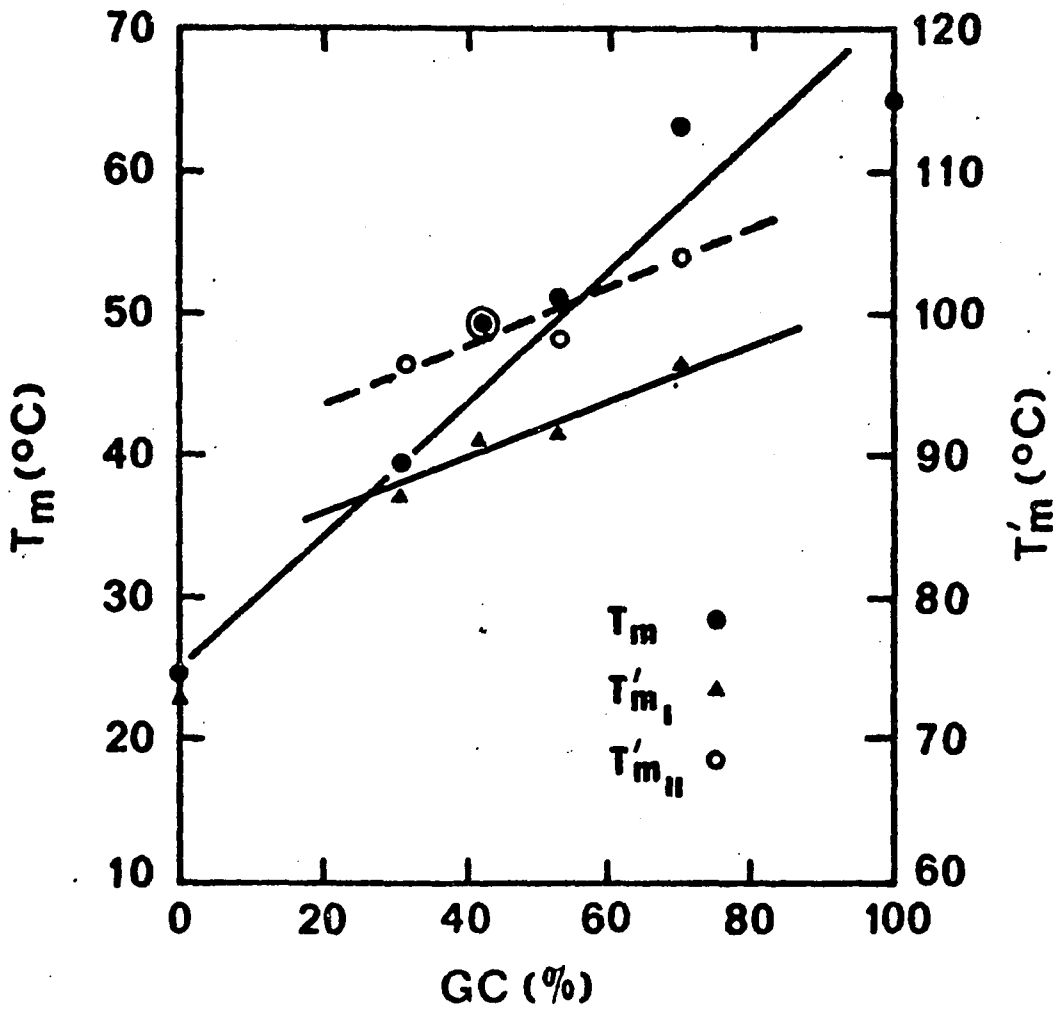


Fig. 6. Dependence of melting temperatures of polyarginine-DNA complexes on the G+C content of DNA.

TABLE IV

The Relation Between the Ratio of Melting Area ($A'_{T_{m,II}}/A'_{T_{m,I}}$) and the G + C Content of DNA

DNA	(G + C)%	$A'_{T_{m,II}}/A'_{T_{m,I}}$
M. luteus	70	0.08
E. coli	53	0.33
Calf thymus	42	0.42
Cl. perfringens	31	0.50

more DNA melting at $T'_{m,I}$ than at $T'_{m,II}$.

(2) Analysis of melting data

Light scattering of these complexes, as measured by A_{320}/A_{260}, is as low as about 0.05 and is not significantly changed before and after melting. Another pertinent melting parameters is h_{max}, the maximum hyperchromicity when melting is completed. As shown in Table V, a complex with a higher r value has a lower h_{max}, a phenomenon also observed in polylysine-DNA complexes prepared by direct mixing (Li et al., 1973). Because the hyperchromicity of peptide-bound regions is different from that of peptide-free regions, Eqs. (6) and (7) in Chapter II are used to determine the β value (average number of amino acid residue per nucleotide in protein-bound regions) and h_b (hyperchromicity of base pairs bound by proteins). Linear plots of Eq. (7) are shown in Figure 7, for polyarginine-DNA, poly (Arg^{87}, Orn^{13})-DNA and protamine-DNA complexes which yield β values of 0.72, 1.05 and 1.38 respectively. In other words, there are 0.72, 1.05 and 1.38 amino acid residues per nucleotide in polyarginine-, poly (Arg^{87}, Orn^{13})- and protamine-bound regions. Linear plots of Eq. (6) are shown in Figure 8 which yield h_b=22.5, 25.0 and 33.6 for the hyperchromicity of base pairs bound by polyarginine, poly (Arg^{87}, Orn^{13}) and protamine.

(3) Effect of ionic strength on the melting curves of complexes

It can be asked whether or not the two melting bands at T'_m region in polyarginine-DNA complexes are due to two different electrostatic neutralization, such that they end up with two different thermal stabilities. This possibility is tested by the experiment shown in Figure 9. A polyarginine-DNA with r=0.45 was made in EDTA buffer and then dialyzed into EDTA buffers plus 0.005, 0.01 and 0.05 M NaCl

TABLE V

The Relation of h_{max} and the Input Ratio of Amino Acid per Nucleotide (r)

r	h_{max} (%)		
	polyarginine-DNA	poly (Arg^{87},Orn^{13})-DNA	protamine-DNA
0.0	35.9	37.0	37.0
0.1	30.2	-	-
0.2	29.6	35.1	-
0.3	32.2	-	36.4
0.4	-	31.9	-
0.45	28.3	-	-
0.6	25.7	30.6	34.7
0.7	23.7	-	-
0.8	-	30.6	-
0.9	-	-	34.5
1.0	-	27.0	-
1.2	-	-	34.0

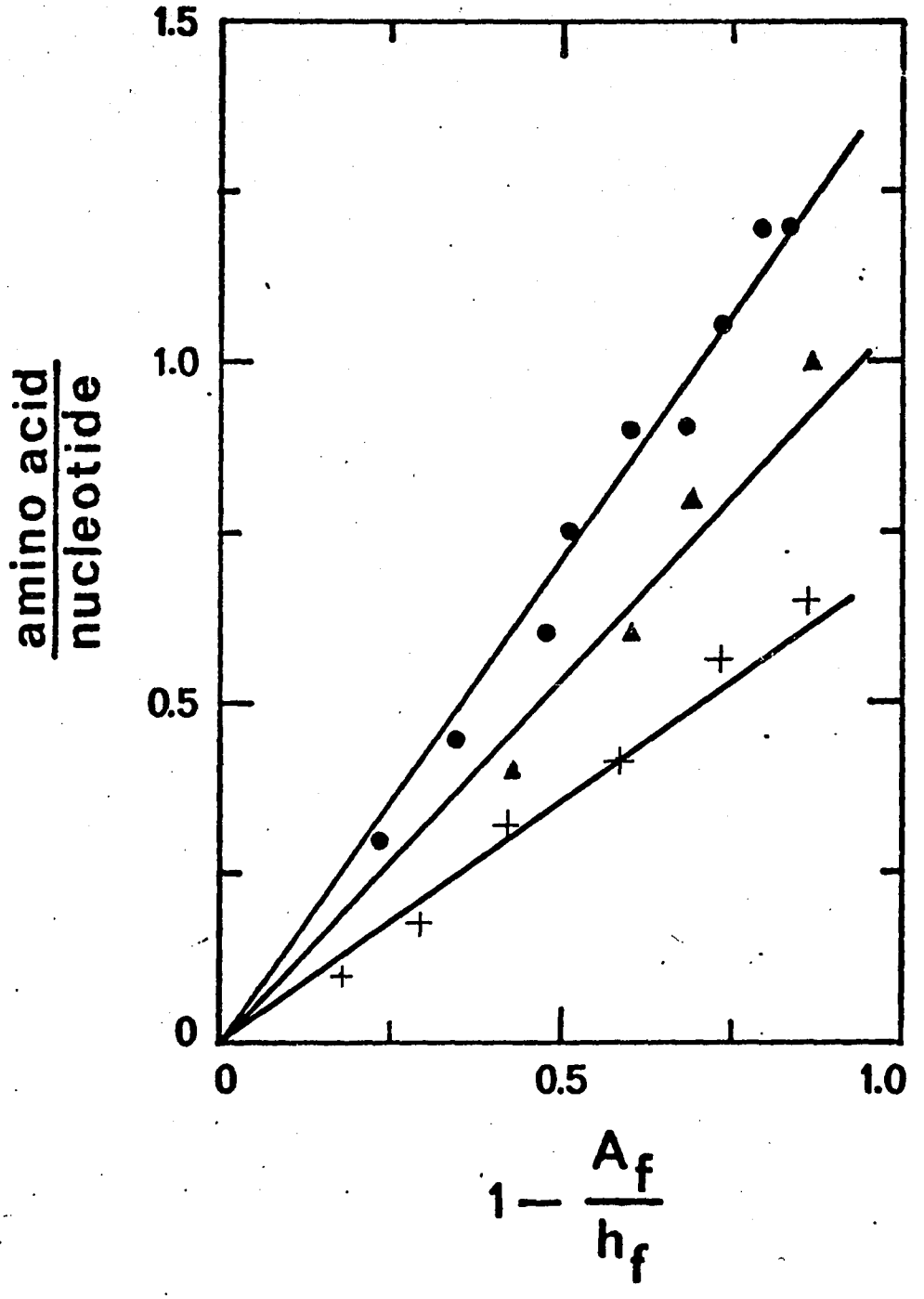


Fig. 7. Linear plots of equation (7) for polyarginine-DNA (+—+), poly $(Arg)^{87}(Orn)^{13}$-DNA (▲—▲) and protamine-DNA (●—●) complexes.

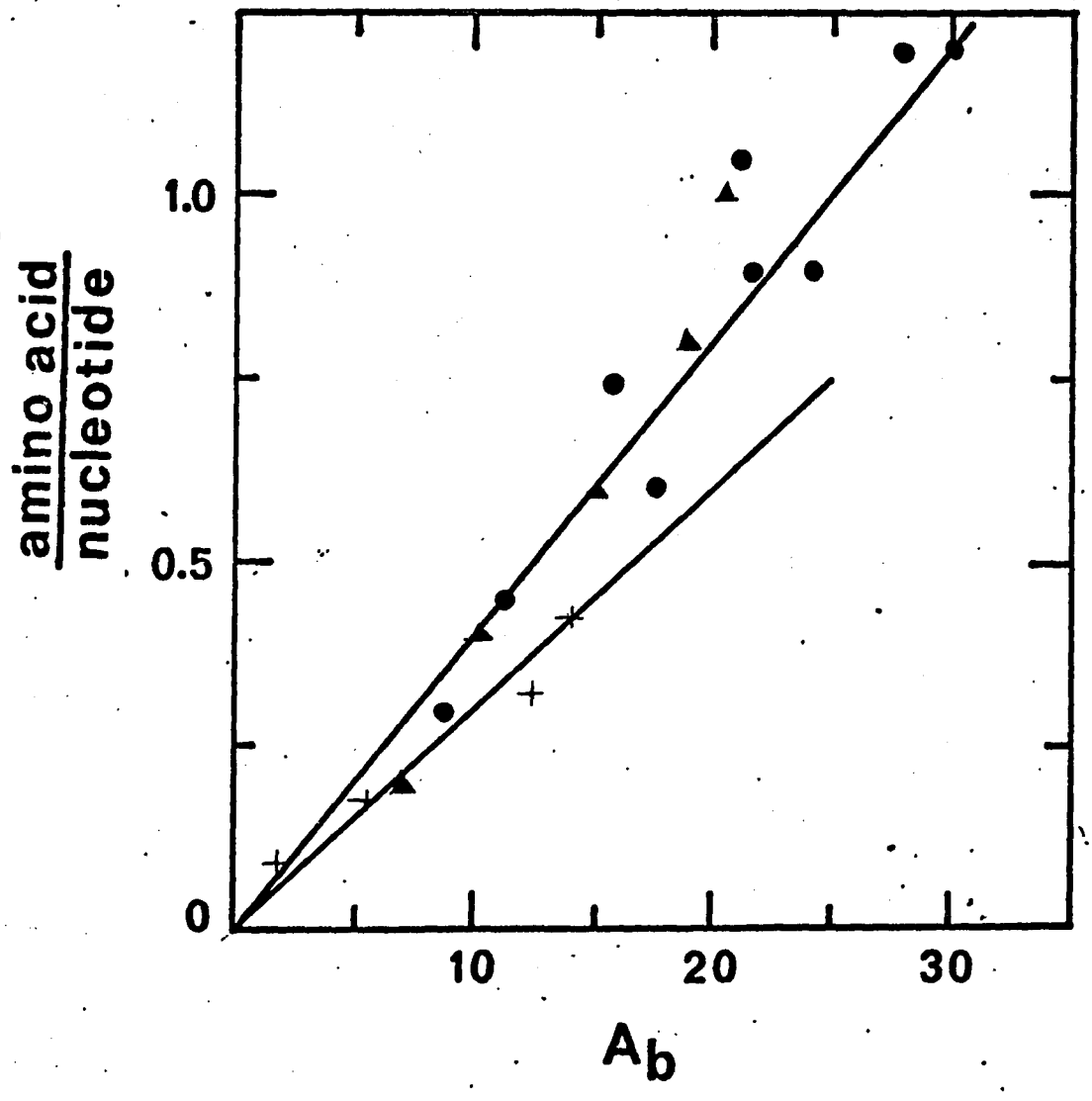


Fig. 8. Linear plots of equation (6) for polyarginine-DNA (+—+), poly $(Arg)^{87}(Orn)^{13}$-DNA (▲—▲) and protamine-DNA (●—●) complexes.

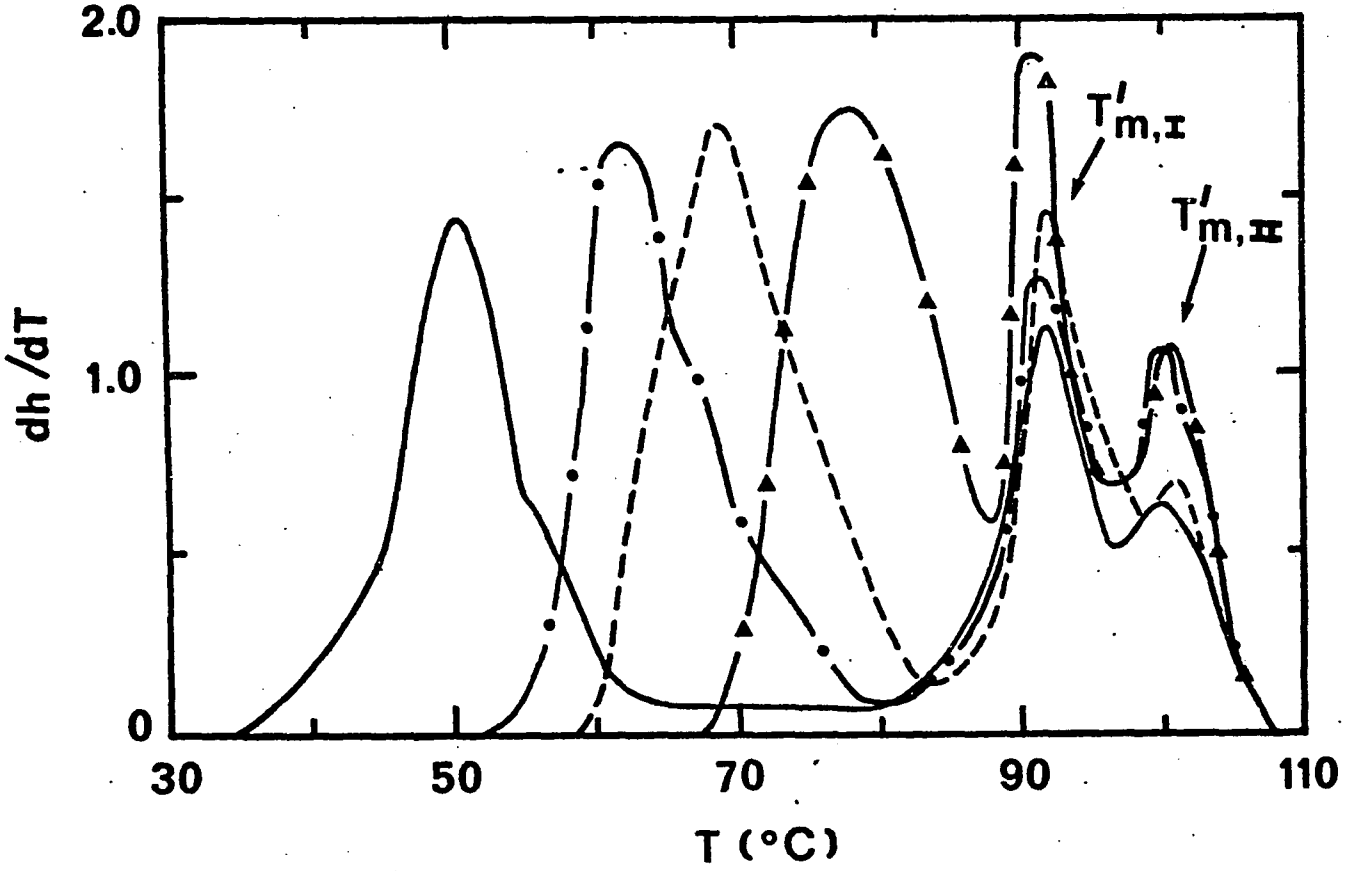


Fig. 9. Effect of ionic strength on melting of polyarginine-calf thymus DNA complex. r = 0.45. The buffer is EDTA (2.5×10^{-4}M) plus NaCl of 0.0M (——), 0.005M (—·—), 0.01M (----) and 0.05M (—▲—).

respectively. The melting temperature of free base pairs at T_m is raised as the ionic strength of the solution is increased. On the other hand, the melting temperatures, $T'_{m,I}$ and $T'_{m,II}$ of bound base pairs are not shifted. These results indicate that electrostatic shielding on those bound base pairs, at $T'_{m,I}$ or at $T'_{m,II}$, are not substantially different.

A β value of 1.38 amino acid residues per nucleotide or 0.92 arginine per nucleotide is found in protamine-bound regions. This indicates that the charges on phosphates of the bound regions are nearly completely neutralized. The effect of ionic strength on melting was also performed to examine electrostatic shielding on DNA (Dove and Davidson, 1962). Figure 10 shows that, as the ionic strength is increased, the T_m of free DNA regions is also shifted to higher temperatures while the T'_m of protein-bound regions remains constant, a phenomenon similar to that found for polyarginine-DNA complexes.

C. Circular Dichroism Studies

Figures 11, 12 and 13 show CD spectra of DNA complexed with various amounts of polyarginine, poly (Arg^{87}, Orn^{13}) and protamine. As more polyarginine, poly (Arg^{87}, Orn^{13}) or protamine is bound to DNA, there are red shifts for the positive band near 275 nm (λ_{max}) and the crossover point near 255 nm (λ_c). The amplitude of the positive band is also reduced. These changes resemble those of DNA in the presence of salt (Tunis-Schneider and Maestre, 1970), in chromatin (Shih and Fasman, 1970) or bound by polylysine (Chang et al., 1973). The CD spectra of DNA complexed with poly (Arg^{87}, Orn^{13}) are similar to those of polyarginine-DNA complexes, though the thermal denaturation results at T'_m are very different in these two complexes.

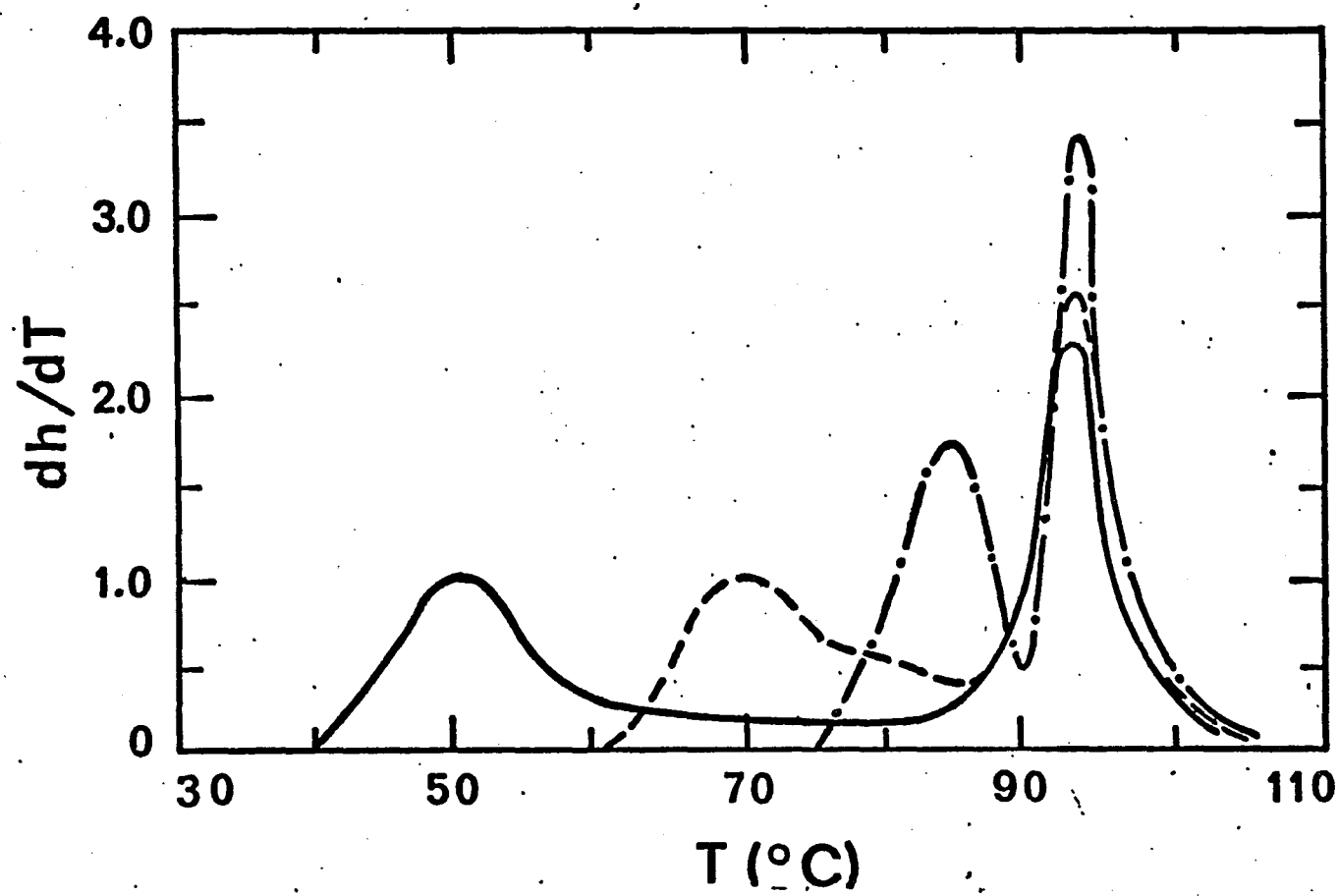


Fig. 10. Effect of ionic strength on melting of protamine-DNA complex (r = 0.8). NaCl concentrations are 7.5×10^{-4}M (——), 1.08×10^{-2}M (---) and 0.1M (—.—).

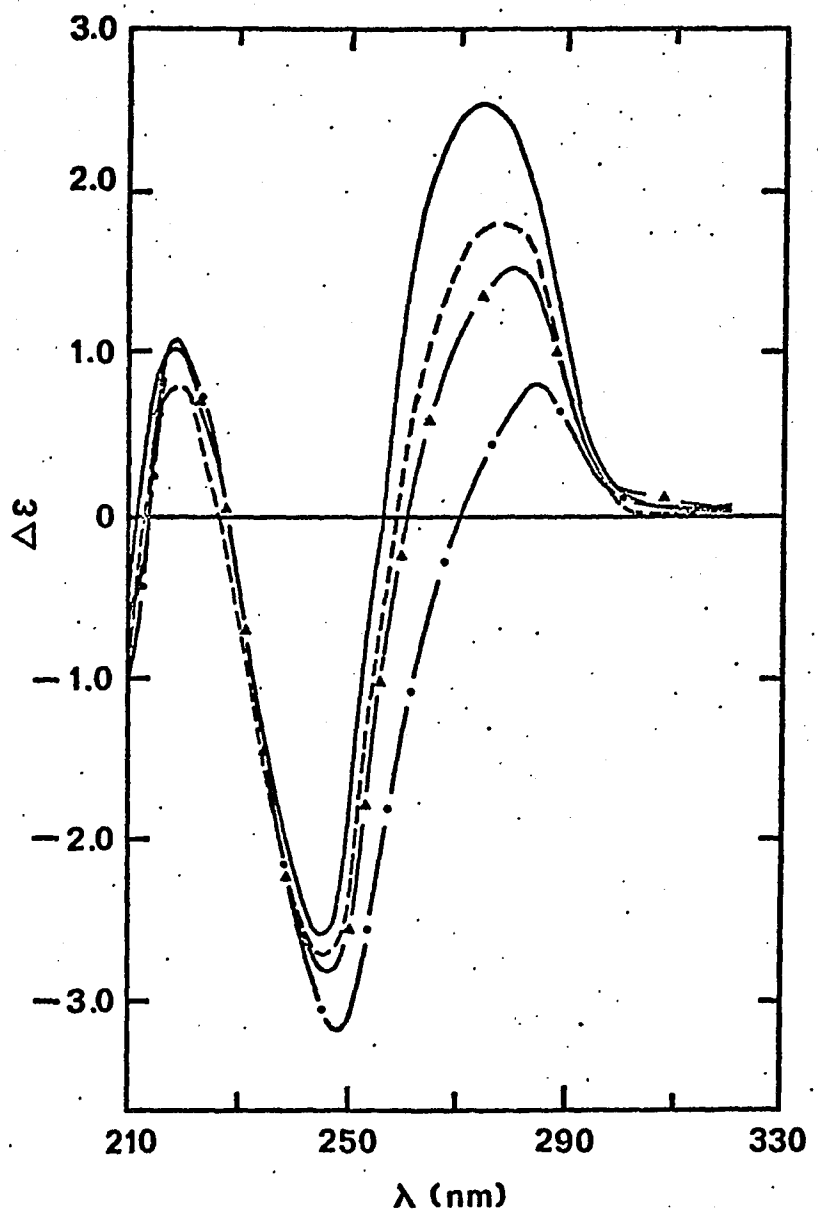


Fig. 11. CD spectra of polyarginine-calf thymus DNA complexes. r = 0 (——), 0.20 (----), 0.35 (—▲—) and 0.60 (—·—).

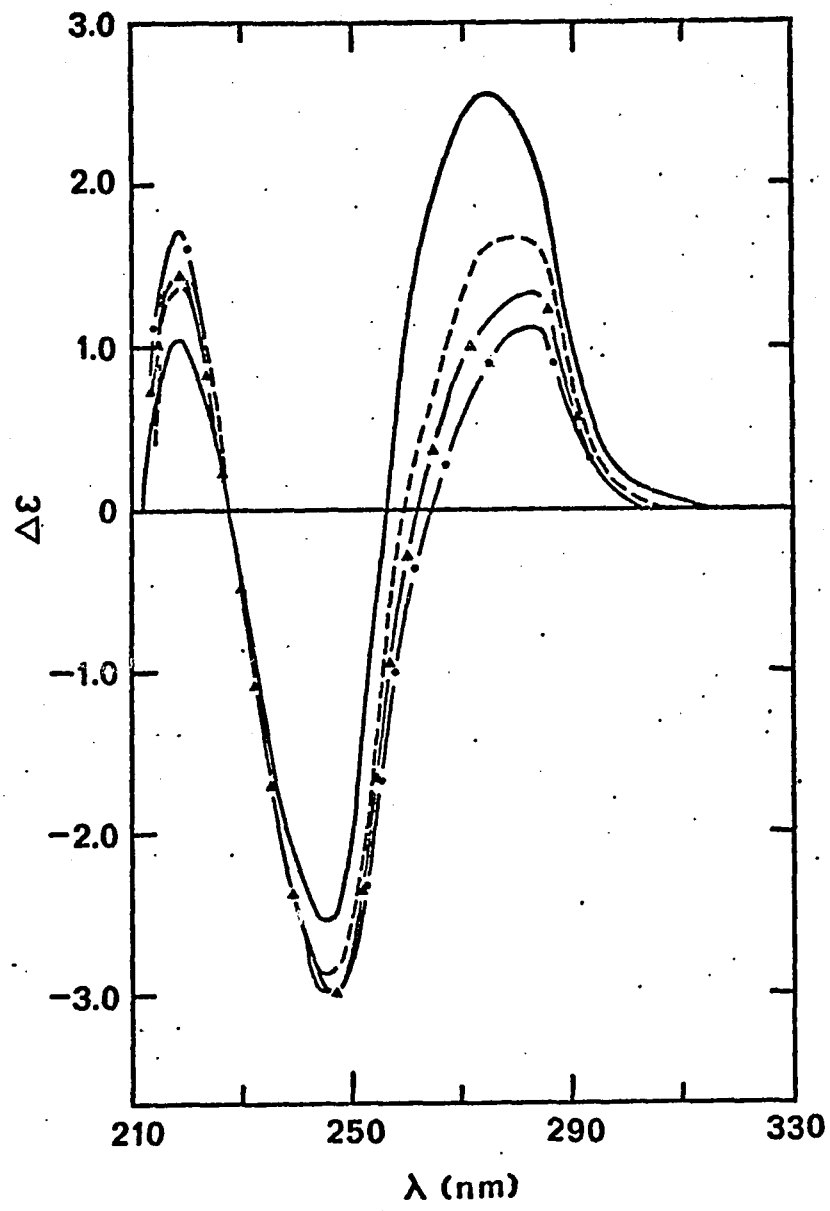


Fig. 12. CD spectra of copoly $(arg)^{87}(orn)^{13}$-calf thymus DNA complexes. r = 0 (——), 0.40 (----), 0.60 (—▲—) and 0.80 (—·—).

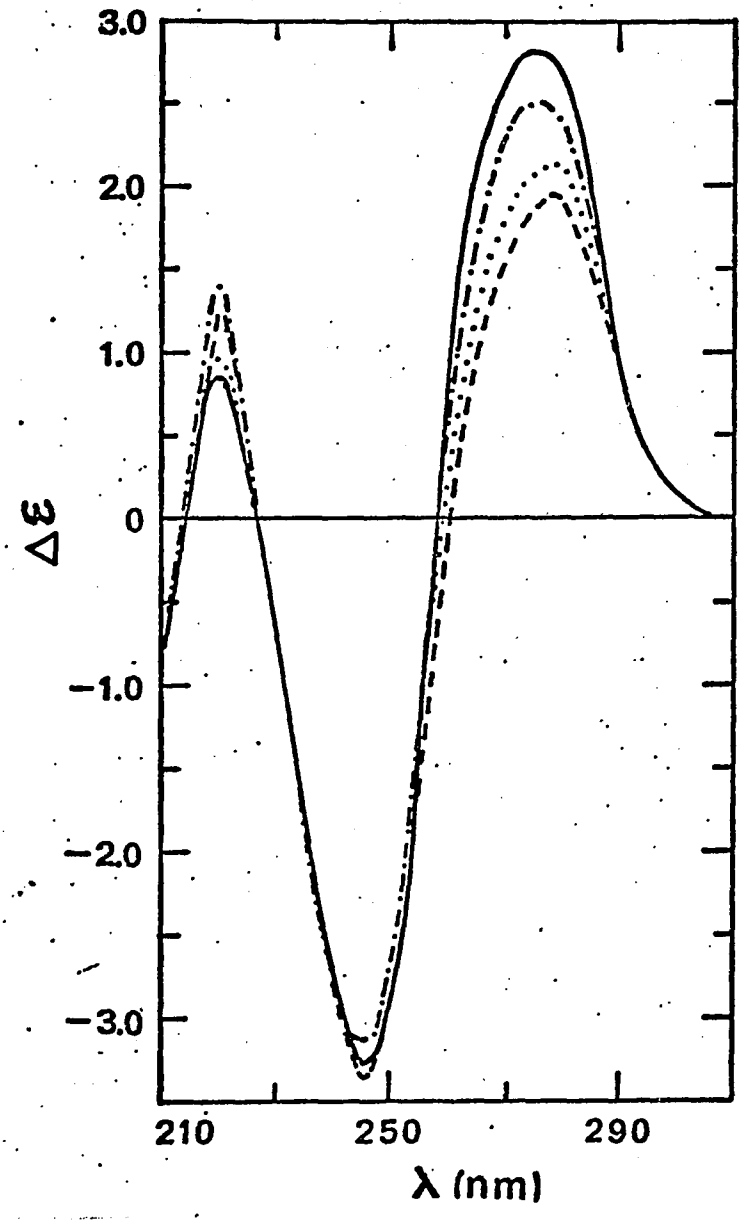


Fig. 13. CD spectra of protamine-DNA complexes. r = 0 (——), 0.3 (—·—), 0.9 (····) and 1.2 (----).

When the difference CD spectra of pure DNA and the complexes were taken it was found that the shapes are identical and the amplitude of each difference CD spectrum is proportional to the r value in that complex. The difference CD spectra of DNA and protamine-DNA complexes are shown in Figure 14 as an example. It indicates that the CD of the complex, $\Delta\epsilon_m$, can be decomposed into two components, $\Delta\epsilon_f^D$ of free and $\Delta\epsilon_b$ of bound base pairs. $\Delta\epsilon_b$ was calculated according to Eq. (9) in Chapter II where F is determined by thermal denaturation method using Eq. (5) (F is the fraction of base pairs bound in each complex). $\Delta\epsilon_b$'s of DNA base pairs bound by polyarginine, poly (Arg^{87}, Orn^{13}) and protamine are shown in Figures 15, 16 and 17. Within experimental errors, $\Delta\epsilon_b$ obtained from different r values are identical to one another for each kind of complex, which confirms the hypothesis behind Eqs. (8) and (9), namely, that there is $\Delta\epsilon_b$ for protein bound base pairs. The CD characteristics of $\Delta\epsilon_b$ are summarized in TABLE VI. It is seen that $\Delta\epsilon_b$ of poly (Arg^{87}, Orn^{13})-DNA is closer to that of polylysine-DNA (Chang et al., 1973) than that of polyarginine-DNA except that, at 220 nm, $\Delta\epsilon_b$ is much greater for poly (Arg^{87}, Orn^{13})-DNA complexes (Figures 15, 16). This is in agreement with thermal denaturation data that the presence of ornithine makes the complexes appear more close to polylysine-DNA complexes. As shown in Figures 3 and 4, there are two melting bands at T_m' in polyarginine-DNA and poly (Arg^{87}, Orn^{13})-DNA complexes, indicating two types of bound base pairs. For the analysis of CD results of these complexes, the average CD, $\Delta\epsilon_b$, of these two types of bound base pairs is used. In other words, it is assumed that, for each complex, the free base pairs have $\Delta\epsilon_f^D$ equal to that of pure DNA and the bound base pairs have an average CD, $\Delta\epsilon_b$, which

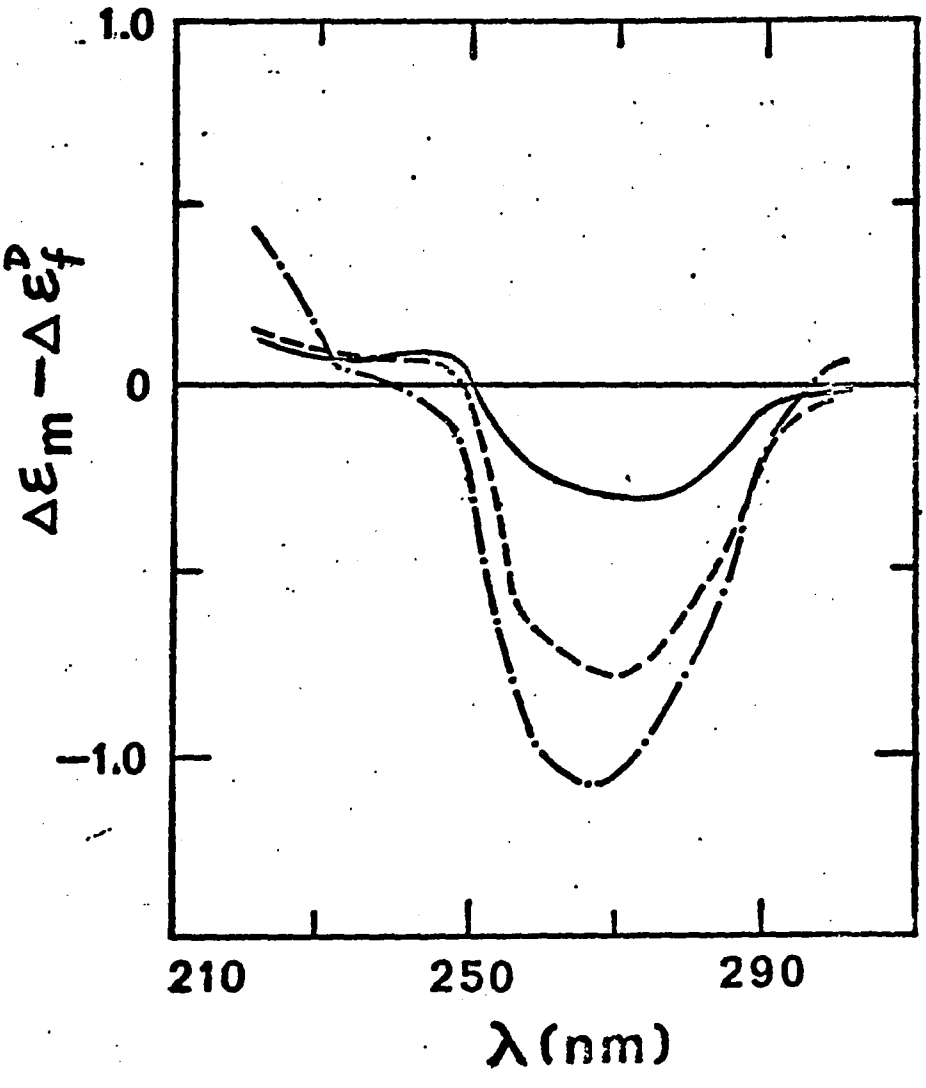


Fig. 14. Difference CD spectra of DNA ($\Delta\varepsilon_f^p$) and protamine-DNA complexes ($\Delta\varepsilon_m$) obtained from Fig. 13. r = 0.3 (——), 0.9 (----) and 1.2 (—·—).

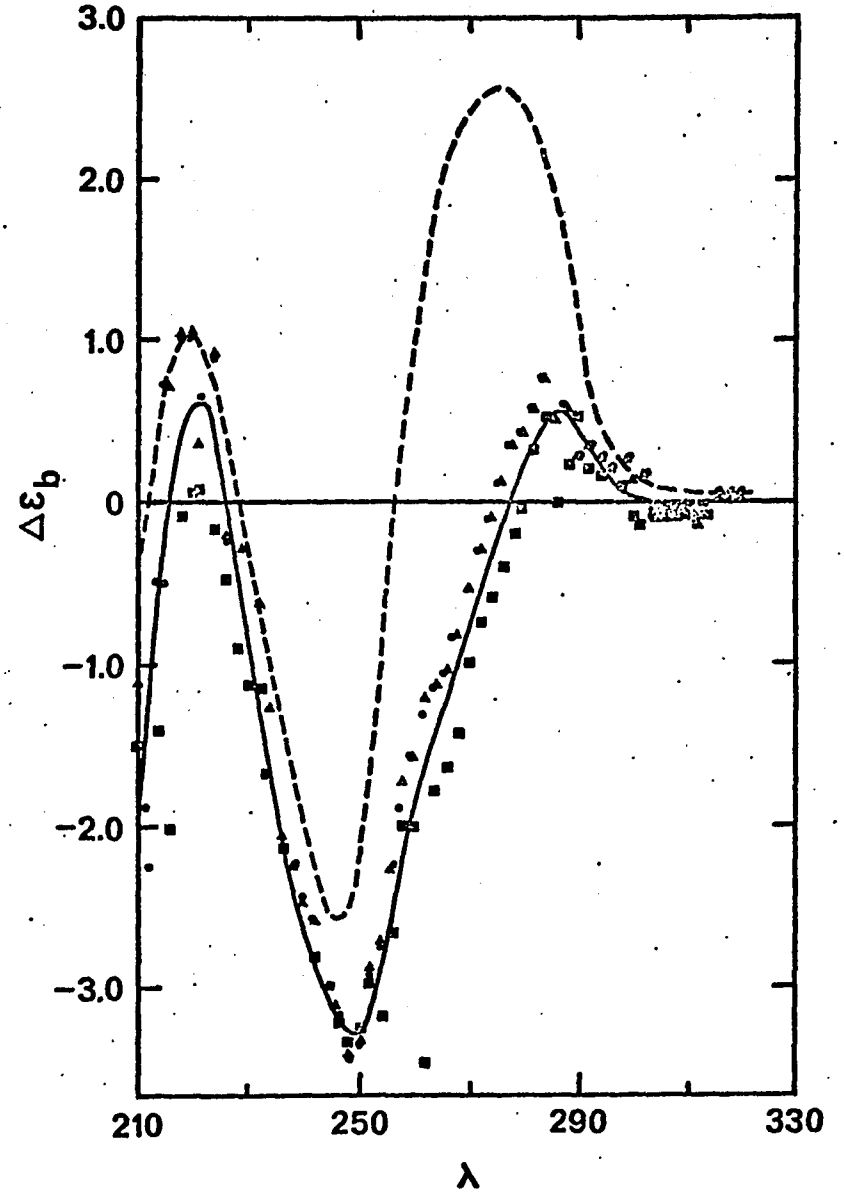


Fig. 15. Calculated CD spectrum ($\Delta\varepsilon_b$) of calf thymus DNA base pairs bound by polyarginine. r = 0.20 (■), 0.35 (▲) and 0.60 (●). Also included is $\Delta\varepsilon_f^p$ of calf thymus DNA (----).

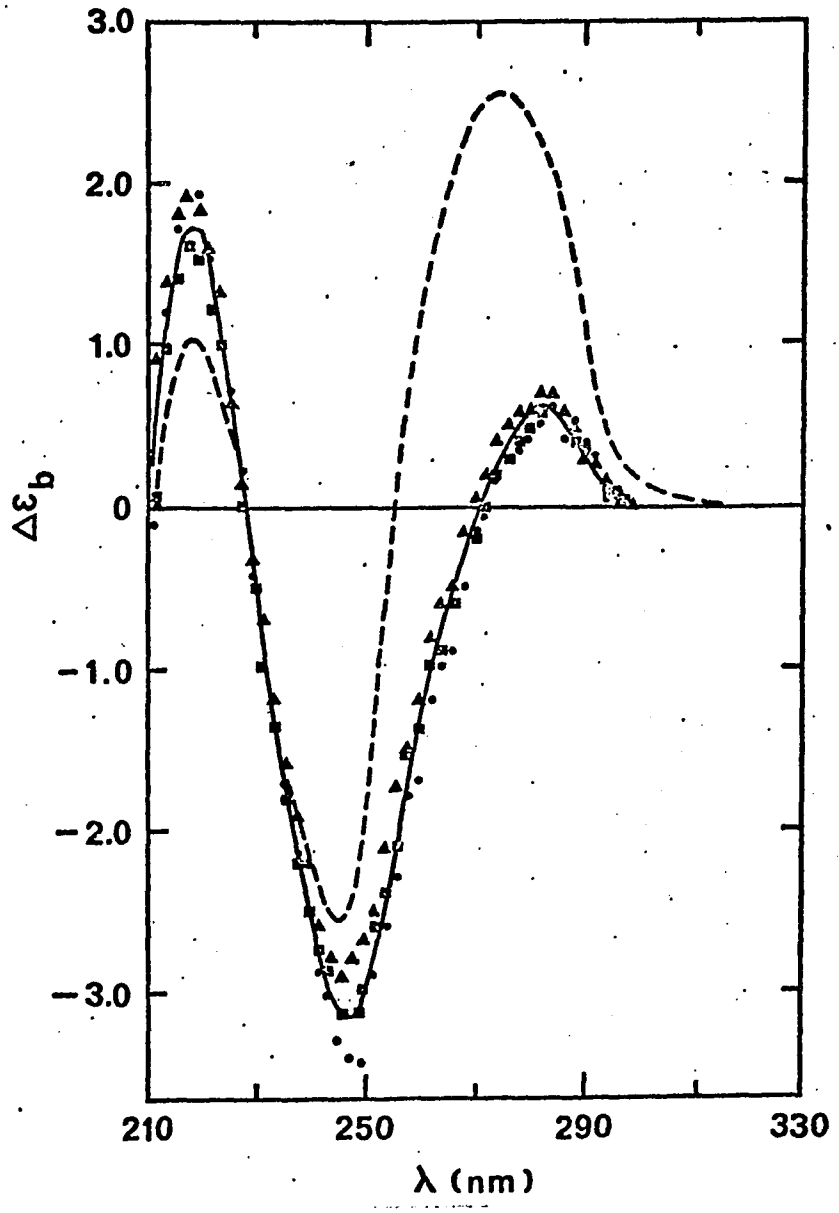


Fig. 16. Calculated CD spectrum ($\Delta\mathcal{E}_b$) of calf thymus DNA base pairs bound by copoly $(arg)^{87}(orn)^{13}$. r = 0.40 (▲), 0.60 (●) and 0.80 (■). Also included is $\Delta\mathcal{E}_f^D$ of calf thymus DNA (---).

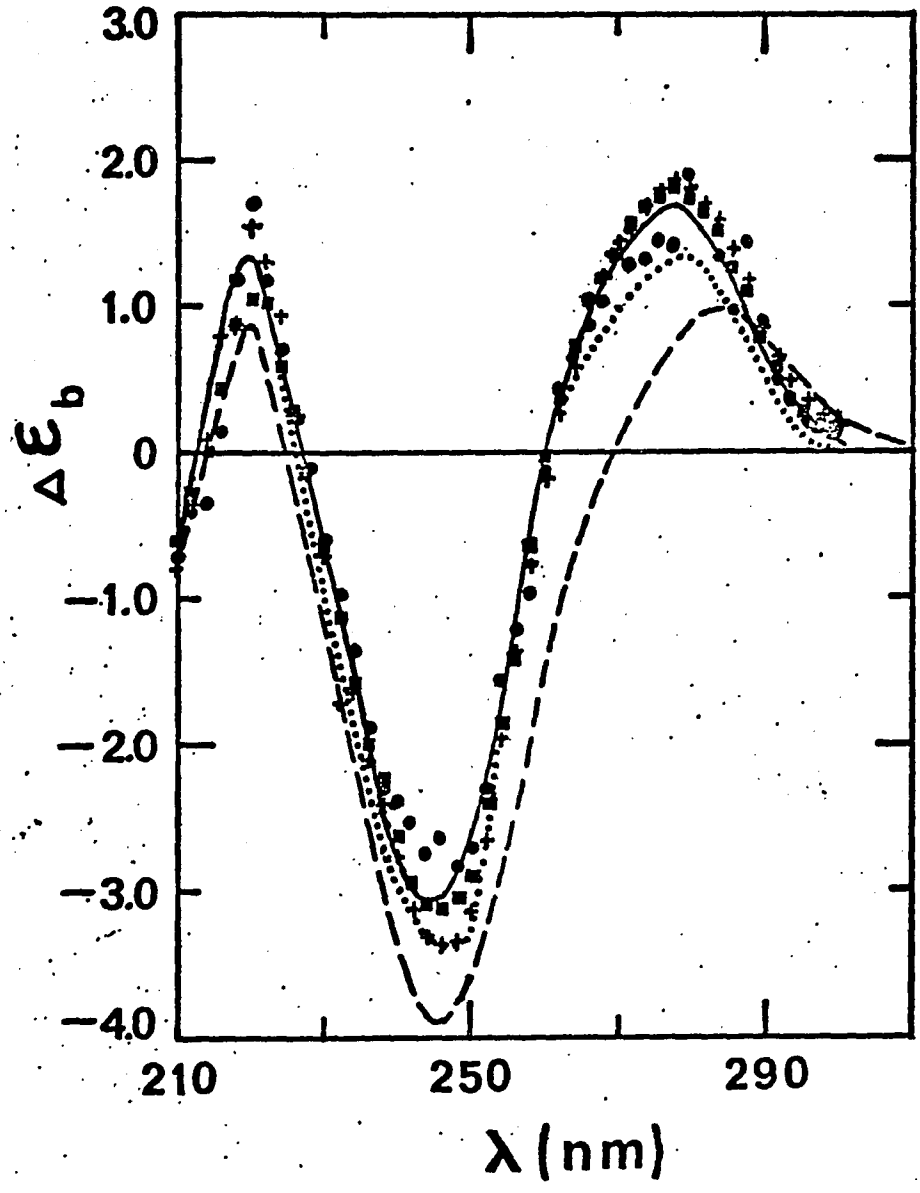


Fig. 17. Calculated CD spectrum ($\Delta\varepsilon_b$) of DNA base pairs bound by protamine (——). r = 0.3 (•), 0.9 (■) and 1.2 (+). $\Delta\varepsilon_b$ of polylysine-bound base pairs (---) (Li et al., 1973) and $\Delta\varepsilon_b$ of DNA in 3.0M NaCl, EDTA buffer (••••) are also included.

TABLE VI

Circular Dichroism Characteristic of $\Delta\varepsilon_b$

	λ_{max}(nm)	λ_c(nm)	$\Delta\varepsilon_{max\lambda}$($M^{-1}cm^{-1}$)
DNA	275	256.5	2.53
polylysine-DNA	283	273	0.80
polyarginine-DNA	286	278	0.53
poly (Arg^{87},Orn^{13})-DNA	282	271	0.63
protamine-DNA	278	261	1.7

is to be determined.

DISCUSSION

Histone H2B with uneven charge distribution along the molecule can induce biphasic melting at T'_m when it binds to DNA (Li and Bonner, 1971). Polylysine with homogeneous charge distribution along the molecule induces only one phase of melting at T'_m (Tsuboi et al., 1966; Li et al., 1973). However, polyarginine which is also homogeneous in charge distribution along the molecule induces biphasic melting at T'_m when it is complexed with DNA (Figure 3). This biphasic melting at T'_m is unexpected.

The melting temperature of polyarginine-bound DNA regions depend on the G+C content of the DNA used (Figure 6). Both $T'_{m,I}$ and $T'_{m,II}$ bear linear relationship with the G+C content of DNA. The relative amplitude of these two melting bands is also a function of G+C content (TABLE IV). $T'_{m,I}$, the lower melting band, is more prominent in a complex with higher G+C content than the one with lower G+C. The latter result is opposite to the possible expectation that the $T'_{m,I}$ corresponds to polyarginine-bound (A+T)-rich regions while the $T'_{m,II}$ to the (G+C)-rich regions.

Figure 9 shows that both $T'_{m,I}$ and $T'_{m,II}$ were not shifted to a higher temperature when the ionic strength of the solution was increased. This indicates that the bound DNA segments, although melted at two temperatures, are electrostatically neutralized. This conclusion is also supported by the similar number of Arg residues per nucleotide (β) in the bound regions for DNA of different G+C contents (Epstein et al., 1974).

Substitution of Arg residues in polyarginine also leads to a change in the relative amplitude of $T'_{m,I}$ and $T'_{m,II}$. The presence of 13% randomly substituted Orn residues in polyarginine results in a shift of the ratio

$A'_{T_{m,II}}/A'_{T_{m,I}}$. The ratio for polyarginine-calf thymus DNA complex is 0.43 while that of poly (Arg^{87}, Orn^{13})-calf thymus DNA complex is 2.9. The presence of Orn also increased the β values from 0.72 for polyarginine to 1.05 for poly (Arg^{87}, Orn^{13}).

Protamine, containing 67% Arg residues and 33% non-basic residues, when bound to DNA, results in only one melting band at 92° corresponding to the $T'_{m,I}$ of polyarginine-DNA complex. No melting band corresponds to the $T'_{m,II}$ of polyarginine-DNA complexes. The protamine-bound DNA region is also nearly electrostatically neutralized (Figure 10). A β value of 1.38 amino acid per nucleotide represents about 0.92 Arg residues per nucleotide which indicates that the charges in protamine-bound regions are nearly neutralized.

When a polyarginine binds to DNA it can wind along either the major or the minor groove of DNA. DNA segments bound by similar polyarginine in opposite grooves would have different melting temperatures at T'_m. The probability for polyarginine to wind along either groove could be a function of the G+C content and the conformation of DNA. This probability could also be modified by amino acid substitution in polyarginine. For instance, as the backbone of a polypeptide winds along the groove of DNA, the side chains extend to the phosphates (Tsuboi et al., 1966); the length of amino acid side chain and the secondary structure of DNA could be critical for the selection of the groove for protein binding; chemical environments in the grooves are different and could play a major role for polypeptide binding if interation between amino acid residues and nucleotides (particularly on the bases) are involved.

Our experimental results are in agreement with the above suggestion that polyarginine could bind DNA in both grooves to produce $T'_{m,I}$ and

$T_{m,II}$. In the case of protamine binding, on the other hand, the existence of only $T'_{m,I}$ melting band could possibly imply the binding of protamine in only one of the two grooves of DNA.

CHAPTER IV

PURIFICATION OF HISTONE H3, MONOMER, DIMER AND OLIGOMERS

Published procedures for the separation histone H3 from the other four histone fractions have exploited the fact that the histone H3 is the only Cys containing histone (Hnilica, 1972). Ruiz-Carrillo and Allfrey (1973) have purified calf thymus histone H3 by binding it to an organo-mercurial sepharose column. Histone H3 was recovered from the column by subsequent elution with thiol compounds such as mercaptoethanol or dithiothreitol (DTT). Sanders and McCarty (1972) have purified duck erythrocyte histone H3 by oxidation and subsequent column chromatography. Crude histone H3 was first isolated by the differential precipitation method of Johns (1964) and the histone H3 in the fraction was oxidized to the dimeric form via -S-S- linkage. The dimeric histone H3 was then separated from the other histones by column chromatography on sephadex G-100. This chapter described the adoption of this oxidation method to the purification of histone H3 from calf thymus.

A. Purification of Calf Thymus Chromatin

Calf thymus chromatin was prepared by a modification of the methods of Shih and Bonner method (1969). 50 gms of minced calf thymus were homogenized with a Waring blender in 200 ml of sucrose medium (0.25 M Sucrose, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.0) at "low" setting for 2 minutes. 400 ml of this sucrose medium was added and further blended at "high" setting for 1 minute. The homogenate was filtered through 4 layers of cheesecloth and then through 2 layers of miracloth to remove large fragments and connective tissues. The nuclear fraction was isolated by a low speed centrifugation (2,000 x g for 20 minutes) and washed once with the sucrose medium. The nuclear pellets were lysed by suspending them with a teflon homogenizer in 60 ml of 10 mM Tris-HCl, pH 8.0. Chromatin was recovered by centrifugation at 12,000 x g for 20 minutes. The pellets were washed 2 times by resuspension in 10 mM Tris-HCl, pH 8.0 and subsequent centrifugation at 12,000 x g for 20 minutes. The final washed pellets were then resuspended in 30 ml of Tris buffer. 5.0 ml of this crude chromatin suspension were layered on 25 ml of 1.7 M sucrose, 10 mM Tris-HCl, pH 8.0, in a SW 25 centrifuge tube and centrifuged at 50,000 x g for 2 hours. The sucrose solution was removed by pipetting and the pellets resuspended in 10 mM Tris-HCl, pH 8.0 by homogenization. The last trace of sucrose was removed from the purified chromatin by an overnight dialysis against 10 mM Tris-HCl, pH 8.0.

B. Preparation of Crude Histone H3

Crude histone H3 was prepared from purified calf thymus chromatin according to the method of Johns (1964). Purified chromatin was extracted with 4 volumes of ethanol-1.25 N HCl (4:1, V/V) at $4^{\circ}C$ for 3 hours. The

mixtures were then centrifuged at 12,000 x g for 30 minutes. The supernatant was then dialyzed overnight against absolute ethanol at 4°C. The precipitates formed were then centrifuged down, washed first with ethanol, and then with acetone, and finally dried under vacuum. The crude histone H3 fractions as prepared by this method still contained large amounts of contaminating histones H2A and H4 as determined by electrophoresis.

C. Oxidation of Crude Histone H3

Separation of histone H3 from contaminating histones was achieved by taking advantage of the cysteine content of histone H3. Calf thymus histone H3 contains two -SH groups and can be oxidized to the dimeric and oligomeric forms by inter-molecular -S-S- bond formation (Panyim et al., 1971). These forms, by virtue of their high molecular weights, can be easily separated from histone H2A and H4 by gel filtration in Sephadex G-100. Intra-molecular -S-S- bond can also occur during oxidation forming the cyclic monomeric histone H3. The cyclic form of histone H3 however can not be separated from H2A and H4 in sephadex. Sanders and McCarty (1972) have purified histone H3 from duck erythrocytes by this oxidation method. In this case only a dimer is formed since duck erythrocyte histone H3 contains only one -SH group.

(1) Oxidation of duck crude histone H3

Oxidation of duck histone H3 was performed as described by Sanders and McCarty (1972). Duck erythrocyte H3 + H2A histones, a gift of Dr. L. Sanders, was dissolved in 6 M guanidine-HCl, 0.3 M Tris-HCl, pH 8.3 at a protein concentration of 4 mg/ml. Air oxidation of the -SH groups was achieved by a slow, constant shaking at 37°C for 18 hours. After oxidation, the histone solution was dialyzed overnight against distilled water at 4°C

and precipitated by the addition of 0.67 volume of cold 50% TCA. The precipitated histone was collected by centrifugation and washed with acidified acetone (0.5 ml of 12N HCl per liter of acetone), followed by acetone and ether. After drying in vacuum, the oxidized crude histone H3 was dissolved in 0.1 N HOAc and lyophilized.

(2) Gel filtration of duck histones

11 mg of oxidized duck histones H3 and H2A were dissolved in 0.25 ml of 0.01 N HCl and applied to a Sephadex G-100 column (1.5 x 100 cm) which was preequilibrated with 0.01 N HCl. The column was eluted with 0.01 N HCl and 3 ml fractions were collected. The absorbance of each fraction at 235 nm were then measured. Fractions corresponding to histone H3 dimer and H2A were then pooled and lyophilized. The dried histones were redissolved in 0.1 N HOAc and dialyzed against 0.1 N HOAc. After dialysis the histone fractions were again lyophilized. Figure 18 shows typical run of duck oxidized histones H3 + H2A in Sephadex G-100. The two main protein peaks corresponding to histone H3 dimer and H2A were clearly separated. Such column fractionations were highly reproducible. The complete separation of histones H3 and H2A was further assessed by polyacrylamide gel electrophoresis. Figure 19a shows a gel scan of an oxidized sample of H3 + H2A. As shown in this gel a slight amount of histone H3 monomer is still present. Figures 19b and 19c show the electrophoretic patterns of the separated fractions from Sephadex chromatography. The histone H3 dimer is virtually free of contaminating histones.

(3) Oxidation of calf thymus crude histone H3

50 mg of crude calf thymus histone H3 were oxidized in guanidine-HCl by the method of Sanders and McCarty (1972) and applied to a Sephadex

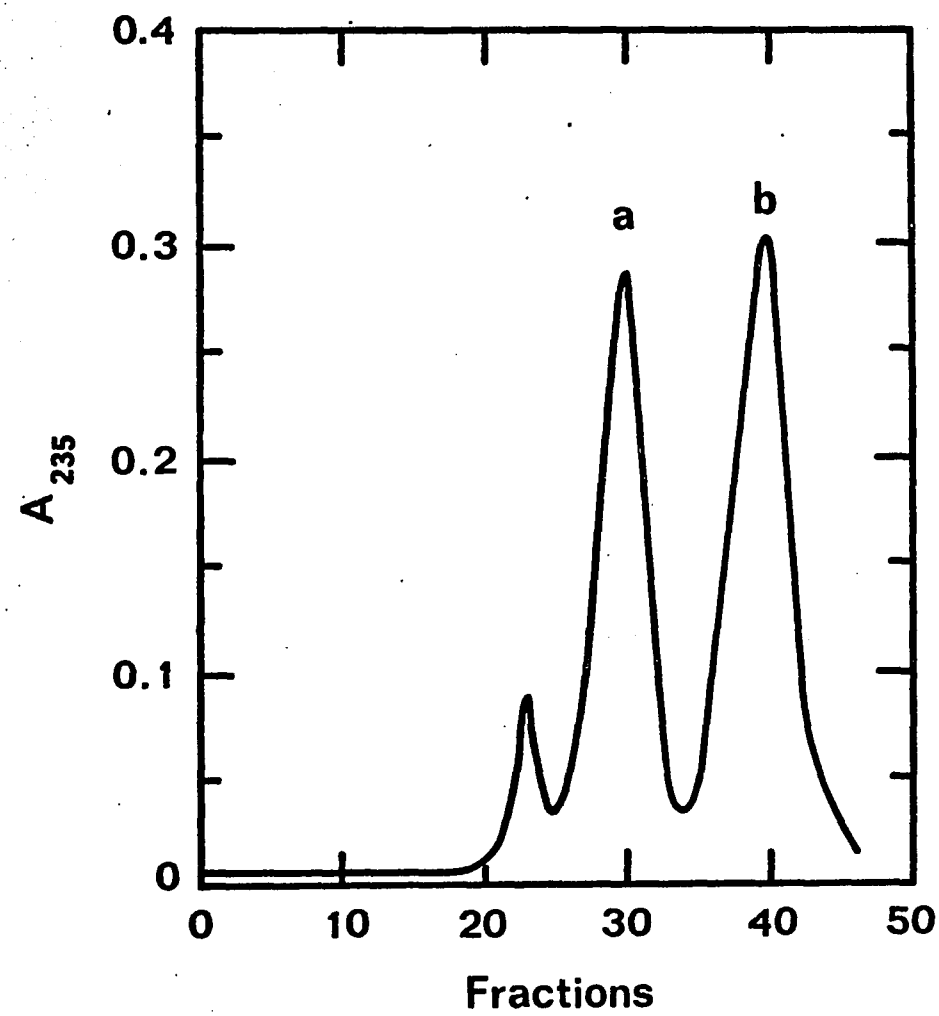


Fig. 18. Gel filtration of duck histone H3 and H2A. 11 mg of oxidized duck histone H3 and H2A were fractionated in a Sephadex G-100 column (1.5 x 100 cm) that was preequilibrated with 0.01 N HCl. 3 ml fractions were collected and their absorbance at 235 nm were measured. Peak a corresponds to histone H3 dimer and peak b corresponds to histone H2A.

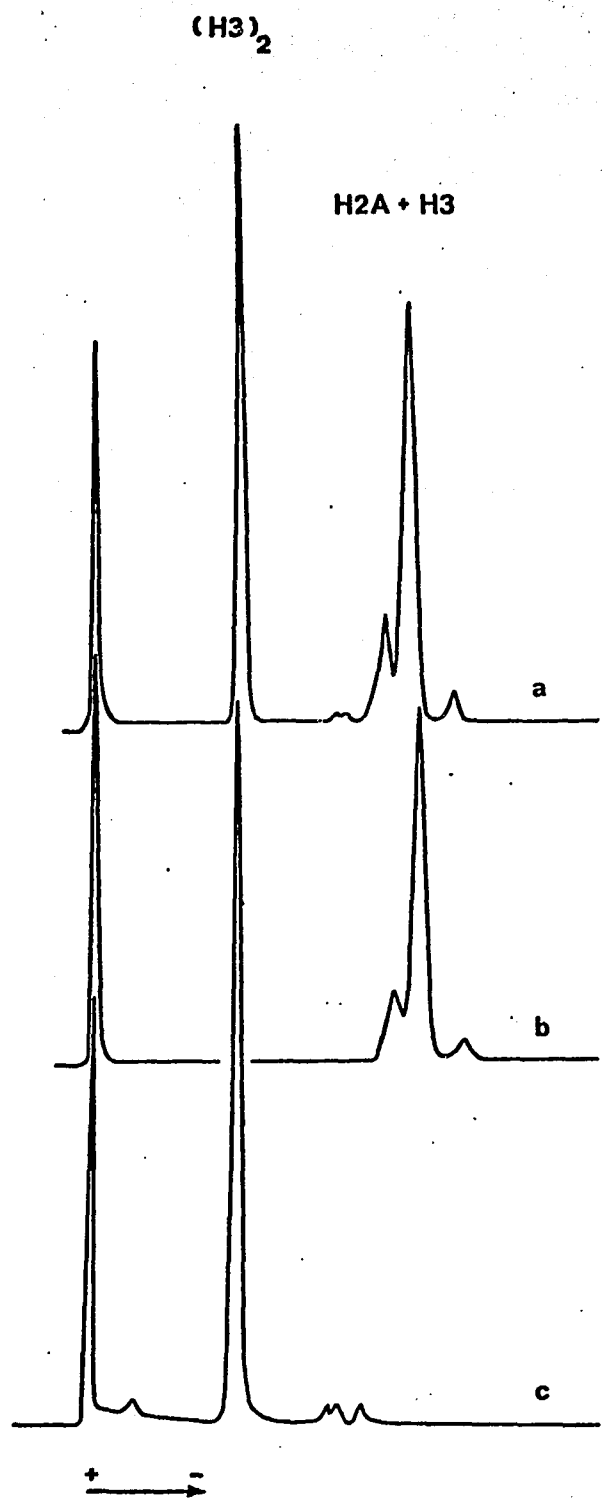


Fig. 19. Polyacrylamide gel electrophoresis of duck histones. (a) Sample contained unfractionated, oxidized duck histone H3 and H2A. (b) and (c) are, respectively, electrophoretic pattern of peaks b and a histones obtained from Sephadex G-100 chromatography as shown in Fig. 18.

G-100 (4.5 x 90 cm) column. The column was eluted with 0.01 N HCl at 22 ml/hr and 150 drops fractions collected. The resulting elution pattern as depicted in Figure 20 shows that very little dimeric or oligomeric forms were obtained (fraction a). A large broad inclusion peak (fraction b) was found, which, on gel electrophoresis, gave a major band that moves slightly faster than the reduced histone H3 but slower than histone H2A. This band corresponds to the cyclic histone H3 monomer as reported by Panyim et al., (1971), utilizing 6M urea in the oxidation medium.

The guanidine-HCl method of oxidation, although useful in the isolation of dimeric form of histone H3 from duck, is not suitable for calf thymus histone H3. Different methods of oxidation have been tried to obtain a good yield of dimeric and polymeric histone H3. Oxidation of histone H3 in the presence of 6M urea, as is the case in guanidine-HCl, results in the rapid formation of intra-molecular disulfide bonds. Oxidation in acidic medium (0.9 N HOAc) results in the formation of dimers, but the rate of reaction is very slow (Panyim et al., 1971). The most efficient and simple method for the formation of histone H3 oligomer is the air oxidation of histone H3 at room temperature in 0.1 M Tris-HCl buffer at pH 8.0 for 8 hours. A typical gel electrophoretic pattern of the oxidized sample is shown in Figure 21. The major oxidized product of histone H3 are the dimeric and oligomeric forms.

Figure 22 shows a typical elution pattern after Sephadex gel G-100 chromatography of this Tris-oxidized crude histone H3 product. In contrast to the chromatographic pattern of guanidine-HCl-oxidized histone H3, two peaks that chromatographed closed to the void volume are well separated from the inclusion peak. Gel electrophoretic patterns of the peak fractions

were d

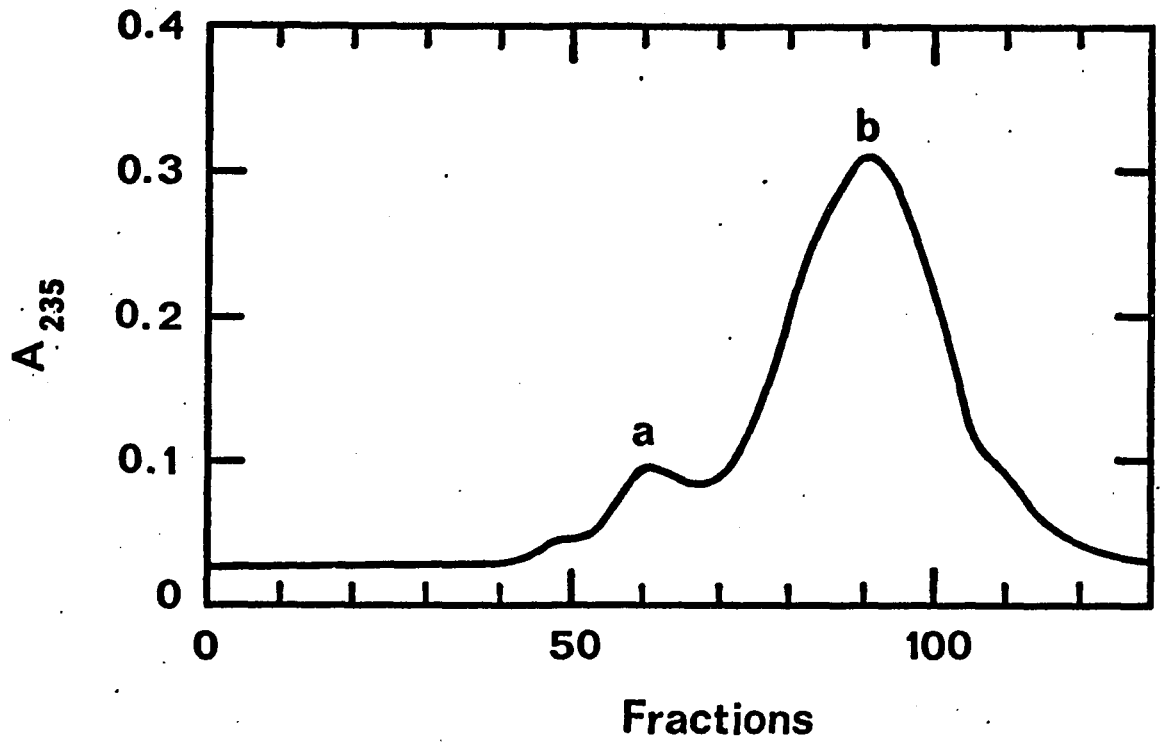


Fig. 20. Gel filtration of calf thymus histone H3 oxidized in guanidine-HCl. 50 mg of crude calf thymus histone H3 oxidized in Gn-HCl were fractionated in a Sephadex G-100 column (4.5 x 90 cm) that was preequilibrated with 0.01 N HCl. The column was eluted with 0.01 N HCl and 150 drops fractions collected. Peak a contains histone H3 dimer and peak b contains both histone H2A and cyclic histone H3.

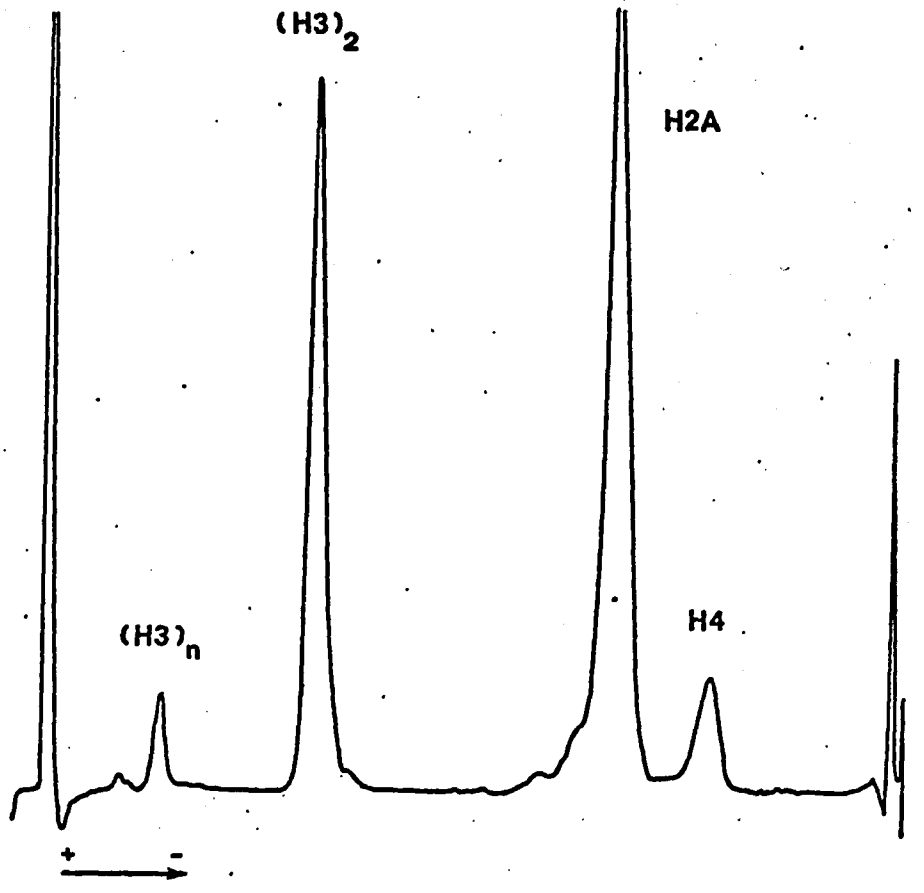


Fig. 21. Polyacrylamide gel electrophoresis of oxidized crude calf thymus histone H3. Crude calf thymus histone H3 was dissolved in 0.1 M Tris-HCl, pH 8.0 (4 mg/ml) and the solution slowly stirred at room temperature for 8 hours. An aliquot of the oxidized sample was then electrophorezed as described in Chapter II. The top cm of the gel containing $(H3)_n$ have multiple protein bands that were not resolved by the scanner.

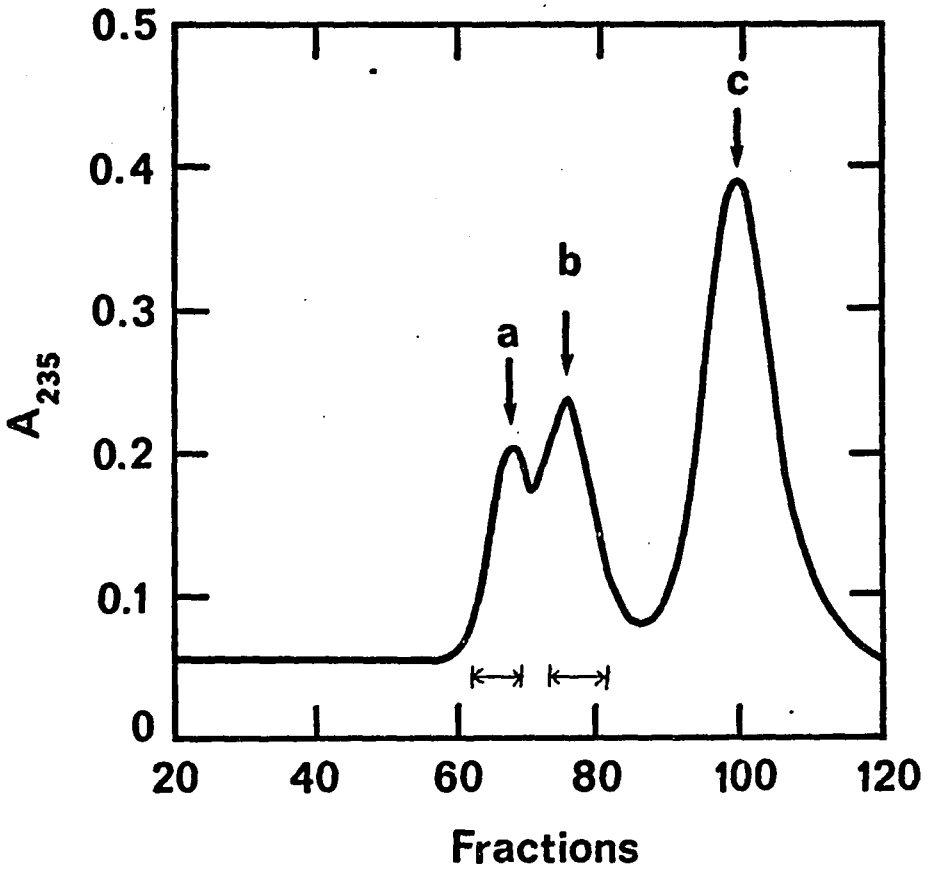


Fig. 22. Fractionation of oxidized crude calf thymus histone H3 by Sephadex G-100 column chromatography. Column size was 4.5 x 90 cm. Histones were eluted by 0.01 N HCl. Fractions a, b and c, respectively, correspond to oligomeric histone H3, dimeric histone H3 and the mixture of H2A, H4 and histone H3 monomer.

were determined. Figure 23a shows that fraction a from the G-100 column contains oligomeric forms of histone H3. Partial reduction of this fraction yields both dimeric and monomeric histone H3. Figure 23b shows that fraction b contains the dimeric form of histone H3. Monomeric histone H3 can be obtained when histone H3 dimer and oligomer are completely reduced with DTT.

(4) Preparation of calf thymus histone H3 dimer and oligomers

Histone H3 dimer and oligomers were pooled following several Sephadex G-100 runs as shown in Figure 22. Fraction a, from tubes 62 to 69, was pooled as oligomeric histone H3 and fraction b, from tubes 73 to 81 was pooled as dimeric H3. Tubes between fractions a and b were pooled and re-chromatographed on Sephadex G-100 column. Similarly tubes after fraction b (82 to 85) were also rechromatographed. Pooled fractions were lyophilized, dialyzed against 0.1 N HOAc and relyophilized.

D. Preparation of Monomeric Histone H3

The oxidized form of histone H3 was reduced by dithiothreitol (DTT) according to the method of Ruiz-Carrillo and Allfrey (1973) to obtain the monomeric form of histone H3. 50 mg of oxidized histone H3 were dissolved in 2 ml of 6 M urea, 40 mM DTT, 0.1 M Tris-HCl, pH 9.0 and incubated at 40°C for 1 hour. Following the incubation, the solution was diluted with an equal volume of water. 50% TCA was then added to give a final concentration of 18%. After centrifugation, the pellet was washed two times with 18% TCA, once with acidified acetone (0.1 ml of 12 N HCl/100 ml acetone), and three times with cold acetone prior to drying in vacuo. A simpler reductive method was also used which gives essentially the same results. Oxidized histone H3 was dissolved in 100 mM DTT at a concentration of 4 to 10 mg/ml and incubated at 37° for 8 hours. The oxidized and reduced

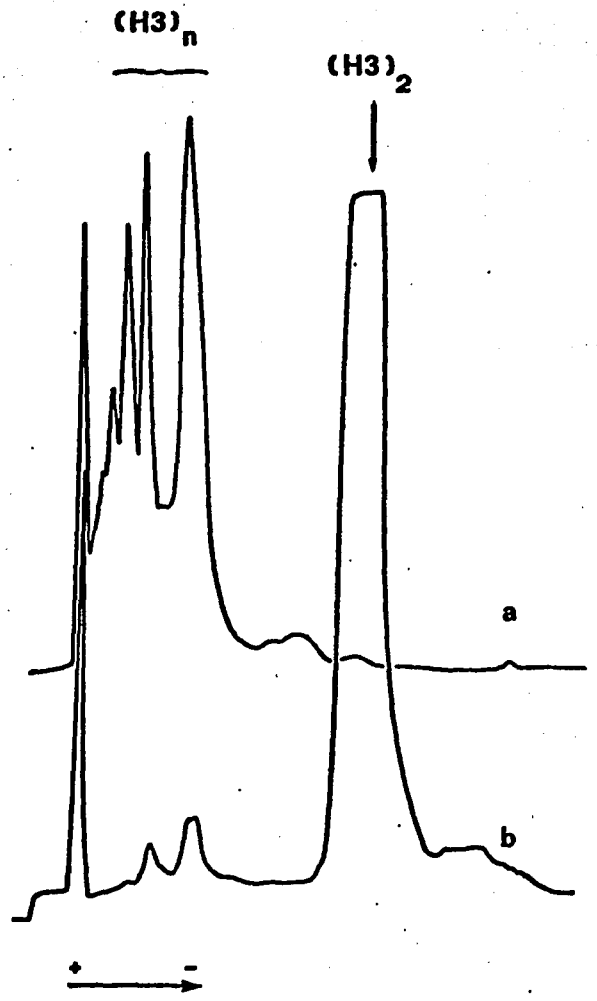


Fig. 23. Polyacrylamide gel electrophoresis of calf thymus histone H3 dimer and oligomers. Gel scans (a) and (b) correspond, respectively, to peak a and b materials from Sephadex G-100 chromatography as shown in Fig. 22. Peak a contain polymeric forms of histone H3 that are larger than a trimer and peak b contain essentially on dimeric histone H3.

form of histone H3 from calf thymus are shown in Figure 24. As shown in the gels more than 90% of the histone H3 was reduced to the monomeric form. Similar results were obtained with duck histone H3.

E. Amino Acid Analysis of Histone H3

Amino acid analysis of purified histone H3, $(H3)_2$ and $(H3)_n$ were kindly performed by Dr. T. H. Liao. Proteins were hydrolyzed in 6N HCl for 18 hours and analyzed in a modified automated amino acid analyzer (Liao et al., 1973). The amino acid compositions for the isolated histone H3, $(H3)_2$ and $(H3)_n$ were normalized with respect to the Ala residue (18 residues/mol). Table VII shows that the amino acid compositions of histone H3, $(H3)_2$ and $(H3)_n$ are similar to each other and also similar to that reported by DeLange et al., (1973) in their sequence work on calf thymus histone H3. Minor differences in the composition were found only in Asp, Thr, Glu and Met content.

F. Discussion

Purification of histone H3 from calf thymus and duck erythrocyte was achieved by first utilizing the differential extraction method of Johns (1964) followed by Sephadex gel chromatography of the oxidized histone H3 to separate it from contaminating lower molecular weight histones.

It was found that different types of calf thymus histone H3 oxidation products could be obtained when different methods of oxidation were utilized. In the presence of denaturating agents such as guanidine-HCl or urea, an intramolecular form predominates while in the absence of such agents mostly dimeric and oligomeric products were obtained. This is

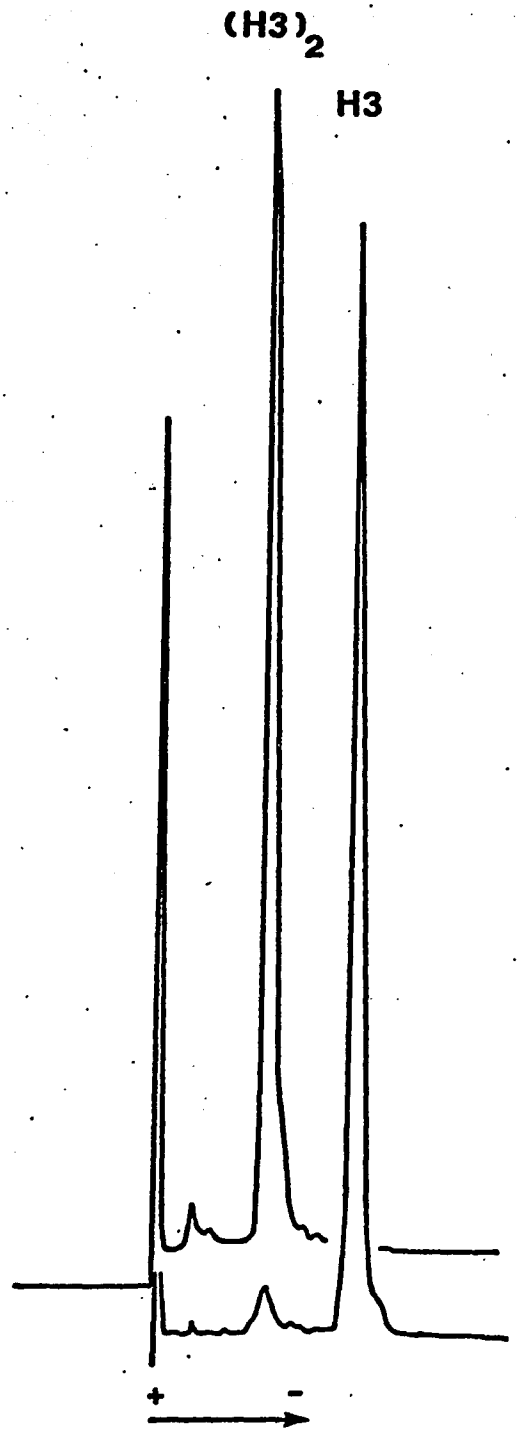


Fig. 24. Polyacrylamide gel electrophoresis of calf thymus histone H3 monomer. Dimeric calf thymus histone H3 (Upper scan) obtained as described in Fig. 21. were reduced by DTT and an aliquot was electrophorezed (Lower scan). More than 90% of $(H3)_2$ were reduced into monomeric form.

TABLE VII

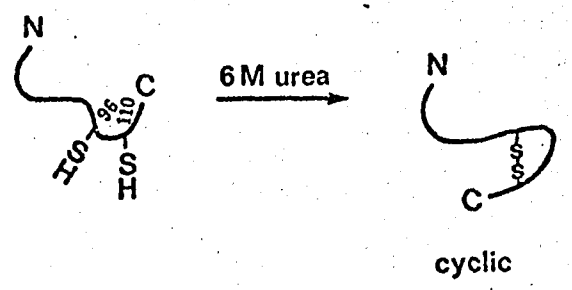
Amino Acid Composition of Histone H3, $(H3)_2$, and $(H3)_n$

Amino Acid	Number of residue per molecule (a)	H3	$(H3)_2$	$(H3)_n$
Asp	5	5.3	6.1	6.2
Thr	10	9.4	9.2	9.3
Ser	5	4.8	4.7	4.7
Glu	15	16.2	15.7	16.3
Pro	6	6.1	5.8	5.7
Gly	7	7.3	7.3	7.4
Ala	18	18	18	18
Val	6	5.9	5.8	6.0
Met	2	2.4	1.6	0.7
Ileu	7	6.7	6.6	6.6
Leu	12	12.3	12.1	12.2
Tyr	3	2.8	2.6	2.8
Phe	4	3.9	3.8	3.9
Lys	13	12.9	12.9	12.8
His	2	1.9	1.9	2.0
Arg	18	17.7	17.6	17.2
Cys	2	(b)	(b)	(b)

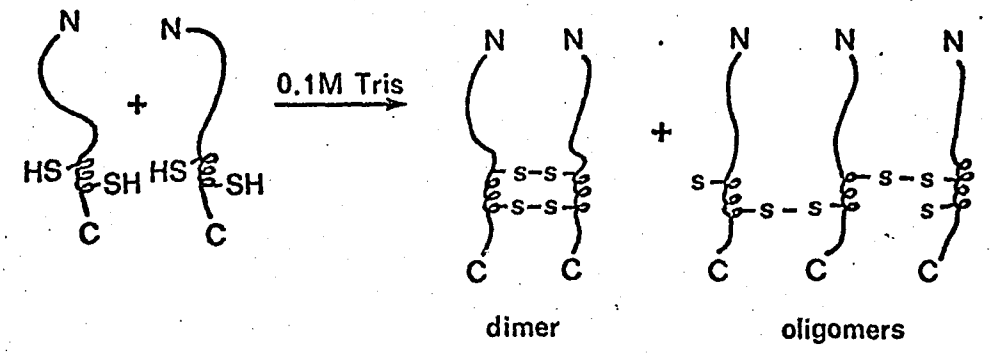
(a) DeLange et al., (1973)
(b) not determined

diagrammatically shown as following:

(a)



(b)



Panyim et al. (1971) have also shown that in the presence of HOAc a very slow oxidation of histone H3 to mainly dimeric form was obtained.

The tendency for the formation of intermolecular disulfide bonds could possibly be explained in terms of the secondary and tertiary structures of histone H3 in the various media. Figure 25 shows the CD spectra of histone H3 in several media. In water, histone H3 shows a negative CD peak at 200 nm and a substantial negative shoulder near 220 nm. It indicates that histone H3 in water contains mostly random coil with some α-helix and/or β-sheet structures. The CD spectra in 0.9 N acetic acid above 220 nm is essentially the same as that in water. In 0.1 M Tris, pH 8.0, the CD spectra becomes more negative between 215nm and 230 nm, suggesting the formation of more α-helix, β-sheet or both. The negative CD above 210 nm is greatly reduced in 6 M urea or in 6 M guanidine-HCl. With regard to secondary structures, histone H3 can be said to contain more ordered structure in 0.1 M Tris than

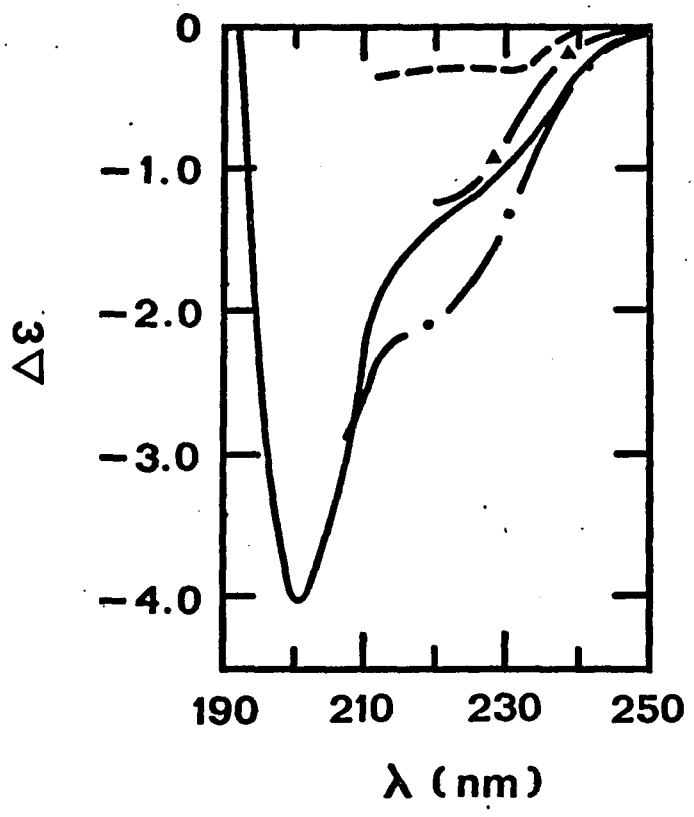


Fig. 25. CD spectra of calf thymus histone H3 in various media. The CD spectra of calf thymus histone H3 monomer dissolved in water (——), 0.1 M Tris, pH 8.0 (— • —), 0.9 N HOAc (—▲—), and 6 M urea (---) were determined.

in water or in 0.9 N acetic acid, and an unordered structure in 6 M urea or guanidine-HCl. The presence of secondary structure seems to increase the efficiency for the formation of histone H3 dimer and oligomers. These results could be explained by the following reasons. (a) When a histone H3 molecule is unordered, such as in 6 M urea or 6 M guanidine-HCl, the C-terminal region is sufficently flexible so that the two -SH groups at position 96 and 110 (DeLange et al., 1973) could form -S-S- disulfide bond more easily than that from the -SH groups of separate histone H3 molecules. Kinetically the former is much more favored than the latter. (b) The formation of some secondary structure, more likely to be in the more hydrophobic C-terminal region, would stiffen this region that the formation of a cyclic histone H3 with intramolecular disulfide bond is sterically hindered. (c) In a medium such as 0.1 M Tris, which favors the formation of secondary structure, it may also favor hydrophobic contact between two or more histone H3 molecules, which would bring the -SH groups from different histone H3 molecules together to facilitate the formation of intermolecular disulfide bond.

CHAPTER V

INTERACTION BETWEEN HISTONE H3 AND DNA

DNA in chromatin has two phases of melting at high temperature due to histone binding (Li and Bonner, 1971; Ansevin et al., 1971). They are 71° and 82° for calf thymus chromatin in 2.5×10^{-4}M EDTA, pH8.0. These two melting bands have been interpreted as due to the binding of the less basic and the more basic regions of histones to DNA (Li and Bonner, 1971).

Circular dichroism (CD) spectrum of DNA in chromatin also differs from that of free DNA. As a result of histone binding the positive CD band of DNA near 275nm is reduced and slightly red-shifted. Histones also contribute a big negative CD band near 220 nm (Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov, 1970; Johnson et al., 1972; Chang and Li, 1974; Wilhelm et al., 1974).

Both thermal denaturation and CD properties of chromatin are therefore well characterized and can be used as criteria to assess the resemblance of a reconstituted chromatin or a histone-DNA complex to that of native chromatin.

In those studies of histone-DNA interaction (Olins, 1969; Fasman et

al., 1970; Wagner, 1970; Shih and Fasman, 1971; Li et al., 1971; Adler et al., 1974; Leffak et al., 1974), it is known that histone-DNA complexes prepared by different methods show very different physical properties. In this chapter both thermal denaturation and CD properties of histone H3-DNA complexes prepared by three different methods were studied and compared to that of native chromatin. The first method utilized a continuous NaCl gradient dialysis (2 M to about 0.1M) in 5 M urea followed by a continuous urea gradient dialysis (5 M to 0 M) and is referred to as gradient dialysis with urea. The second method is a continuous NaCl gradient dialysis (2M to 0.1M) in the absence of urea and is referred to as gradient dialysis without urea. The third method is the direct mixing of histone and DNA solutions in EDTA buffer by slow addition of histone to DNA and is referred to as direct mixing. Calf thymus histone H3 monomer, dimer, and oligomers as well as duck erythrocyte histone H3 monomer and dimer were used in these studies.

RESULTS

A. Complexes Prepared by Gradient Dialysis with Urea

Figures 26a and 26b show, respectively, the thermal denaturation results of histone H3 monomer-DNA and histone H3 dimer-DNA complexes in 2.5×10^{-4} M EDTA. These complexes were prepared by the gradient dialysis with urea method. Although 2 or 3 melting bands may be roughly distinguished for each complex, the peak positions of these bands shifted greatly from one complex to another and is different from preparation to preparation. A higher melting temperature range is found, however, for a complex with a higher value of input ratio of amino acid of histone to nucleotide of DNA (r). These results are different from that obtained from complexes

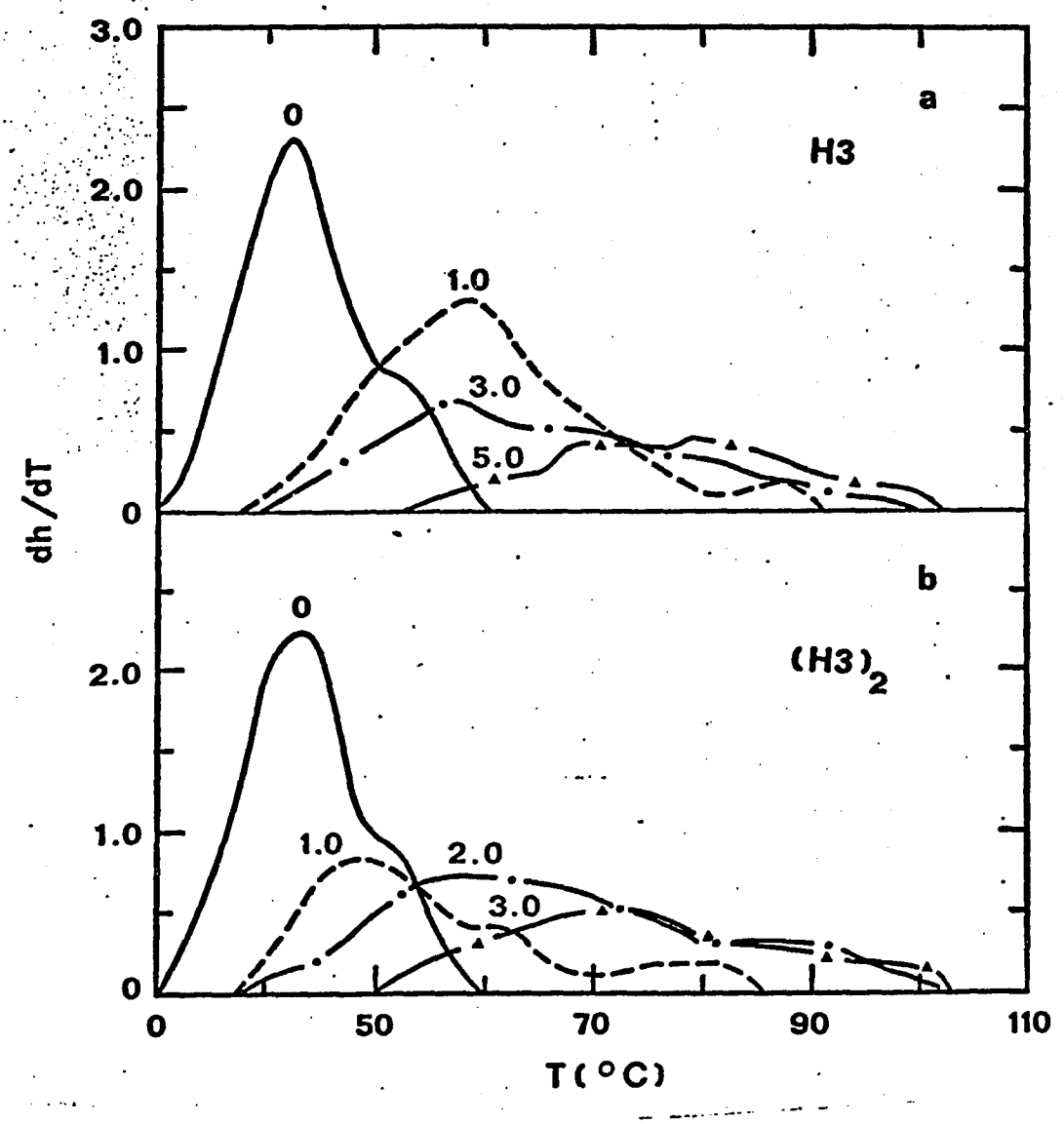


Fig. 26. Derivative melting profiles of calf thymus histone H3-DNA complexes prepared by gradient dialysis with urea. (a) Histone H3 monomer-DNA complexes. r values are 0(——), 1.0 (---), 3.0 (—·—), and 5.0 (—▲—). (b) Histone H3 dimer-DNA complexes. r values are 0 (——), 1.0 (— — —), 2.0 (—·—), 3.0 (—▲—).

of DNA with histone H1 (Shih and Bonner, 1970), H2B (Leffak et al., 1974) or H5 (Hwan et al., 1975) prepared by a similar method of gradient dialysis in urea. In these cases definite melting bands corresponding to histone-free and histone-bound regions can be assigned. Since no definite melting bands can be assigned for histone H3-DNA complexes, the described method (Chapter II) of analyzing melting data is not applicable. Nevertheless, Figures 26a and 26b indicate that both histone H3 monomer-and dimer-DNA complexes are qualitatively similar to each other in their melting properties.

CD results of the two types of complex are shown in Figure 27. There is a proportional decrease in the positive CD band near 275 nm as the r value of the complex is increased. Similarly, a greater red shift of the peak and the crossover point of the band occur in a complex with higher ν value. The reduction of the amplitude is similar to while the red shift is much greater than that in chromatin (Shih and Fasman, 1970). A complex with a higher r value also shows a slightly more negative CD below 235 nm which can be attributed to bound histone. However, the CD contribution near 230 nm of histone H3 in these complexes is much smaller than the histone contribution in the CD spectrum of chromatin. Again, both histone H3 monomer-DNA and dimer-DNA complexes show similar CD spectra.

B. Complexes Prepared by Gradient Dialysis without Urea

The melting profiles of DNA-histone H3 monomer and DNA-histone H3 dimer complexes prepared by the method of gradient dialysis without urea are shown in Figures 28a and 28b respectively. In contrast to the results obtained from complexes formed by gradient dialysis with urea, a definite separation of melting band of histone-free (45-60^{o}) and histone-bound

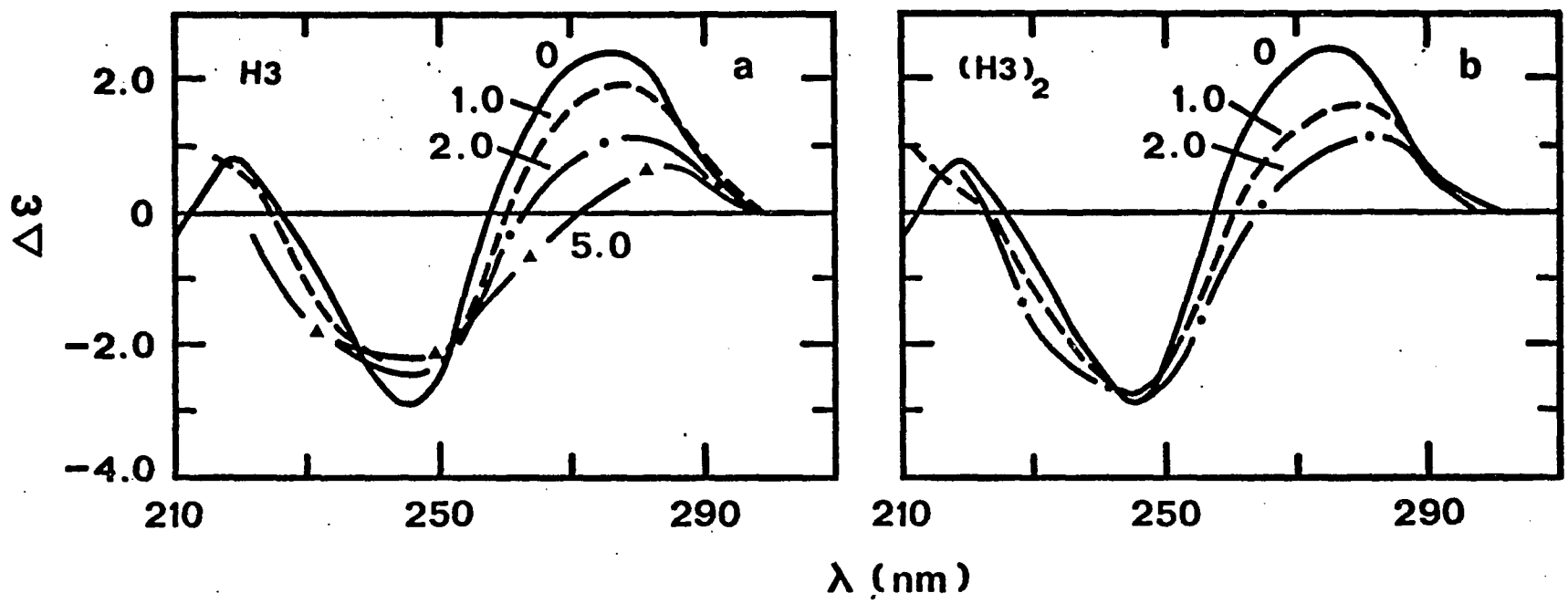


Fig. 27. CD spectra of calf thymus histone H3-DNA complexes prepared by gradient dialysis with urea. (a) Histone H3 monomer-DNA complexes. The r values are 0 (——), 1.0 (---), 2.0 (—·—), and 5.0 (—▲—). (b) Histone H3 dimer-DNA complexes. The r values are 0 (——), 1.0 (---), and 2.0 (—·—).

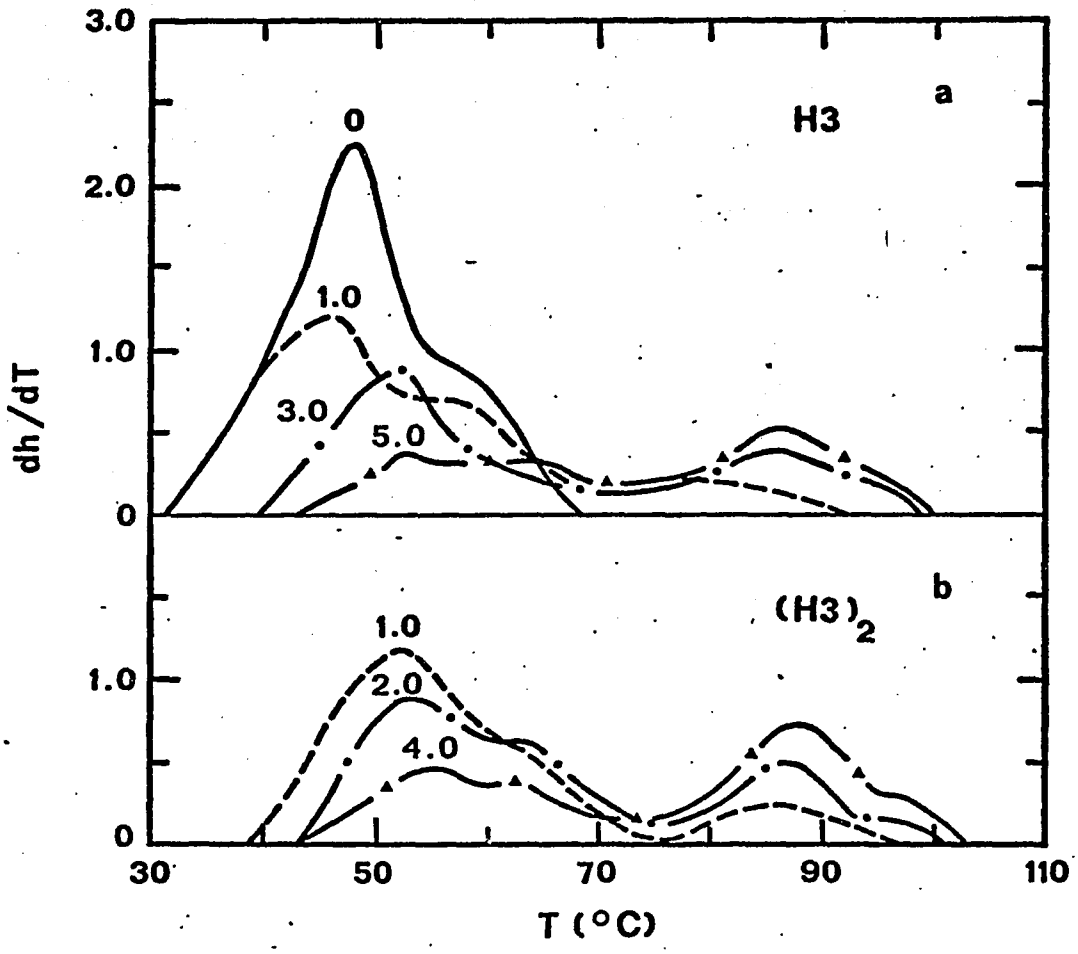


Fig. 28. Derivative melting profiles of calf thymus histone H3-DNA complexes prepared by gradient dialysis without urea. (a) Histone H3 monomer-DNA complexes. The r values are 0 (——), 1.0 (---), 3.0 (—•—), and 5.0 (—▲—). (b) Histone H3 dimer-DNA complexes. The r values are 1.0 (---), 2.0 (—•—), and 4.0 (—▲—).

regions (80-100°) is found. These results indicate that for histone H3 the presence of urea in the reconstitution medium may not be advantageous.

CD results for the complexes of DNA-histone H3 monomer and DNA-histone H3 dimer are shown in Figures 29a and 29b. There is a reduction in amplitude and a red shift of DNA CD band near 275 nm as more histone is bound to DNA. The magnitude of the reduction and red shift is less than that of complexes formed by gradient dialysis with urea (Figure 27). The amplitude of the negative CD band near 220 nm is approximately proportional to r until it reaches 4.0 or 5.0 at which point portion of the complex may have precipitated out of the solution. Compared with the CD results in Figures 27a and 27b for complexes formed by gradient dialysis with urea, complexes formed by gradient dialysis without urea have greater negative CD near 220nm. It implies that there are more secondary structures in the bound histones for complexes prepared by the latter method.

Based upon melting and CD results shown in Figures 26, 27, 28 and 29, the complexes of DNA with either histone H3 monomer or dimer, prepared by gradient dialysis either with or without urea, are approximately identical to each other.

C. Complexes Prepared by Direct Mixing

Melting results of complexes between DNA and calf thymus histone H3 monomer, dimer, oligomers and duck erythrocyte histone H3 dimer prepared by the method of direct mixing are shown in Figure 30. Biphasic melting is evident in all cases, with a melting band near 50° (T_m) corresponding to free base pairs, and another band near 90° (T'_m) corresponding to the histone-bound base pairs. The amplitude of the melting band at T'_m increases with increasing r value in the complex. There is also a

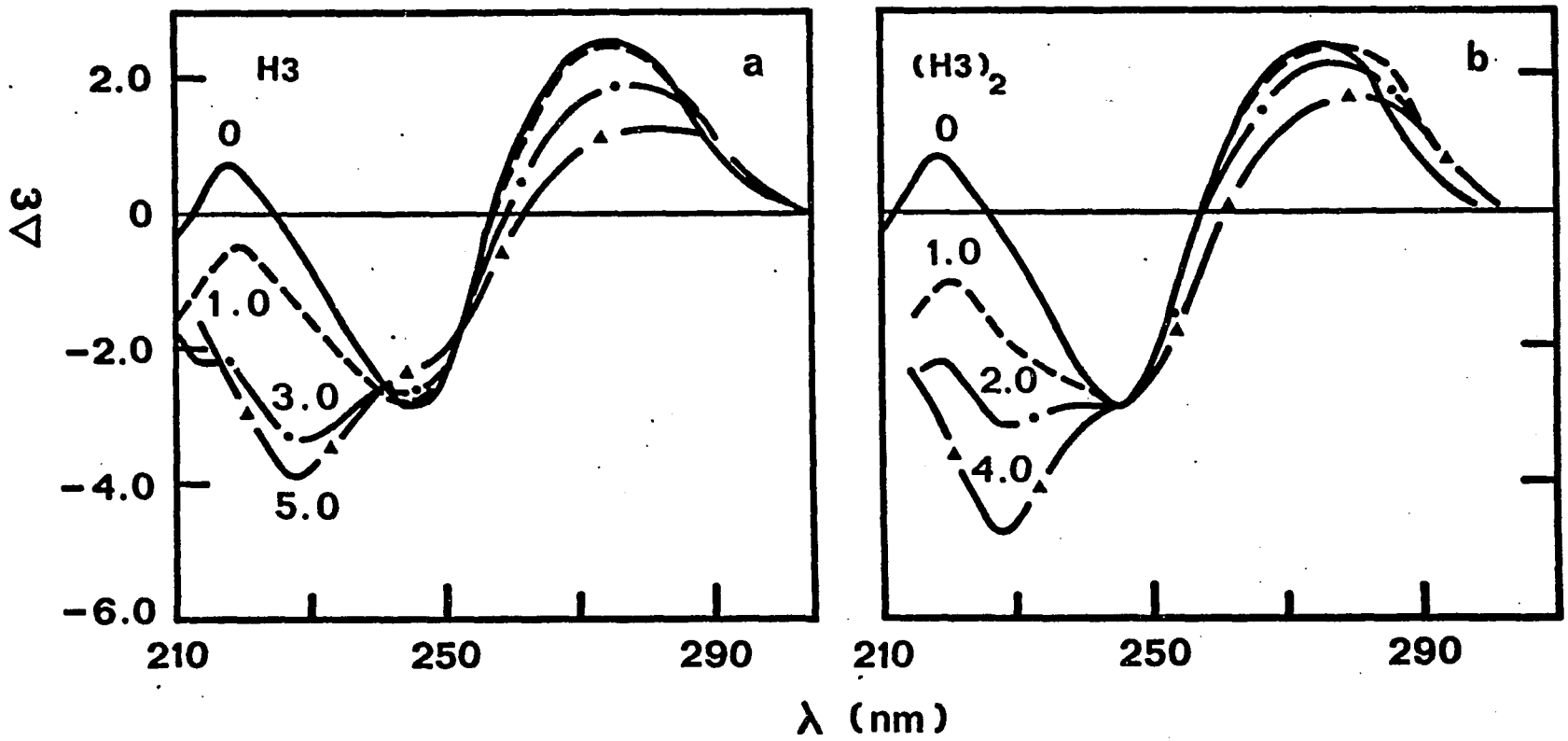


Fig. 29. CD spectra of calf thymus histone H3-DNA complexes prepared by gradient dialysis without urea. (a) Histone H3 monomer-DNA complexes. The r values are 0 (——), 1.0 (---), 3.0 (—·—), and 5.0 (—▲—). (b) Histone H3 dimer-DNA complexes. The r values are 0 (——), 1.0 (---), 2.0 (—·—), and 4.0 (—▲—).

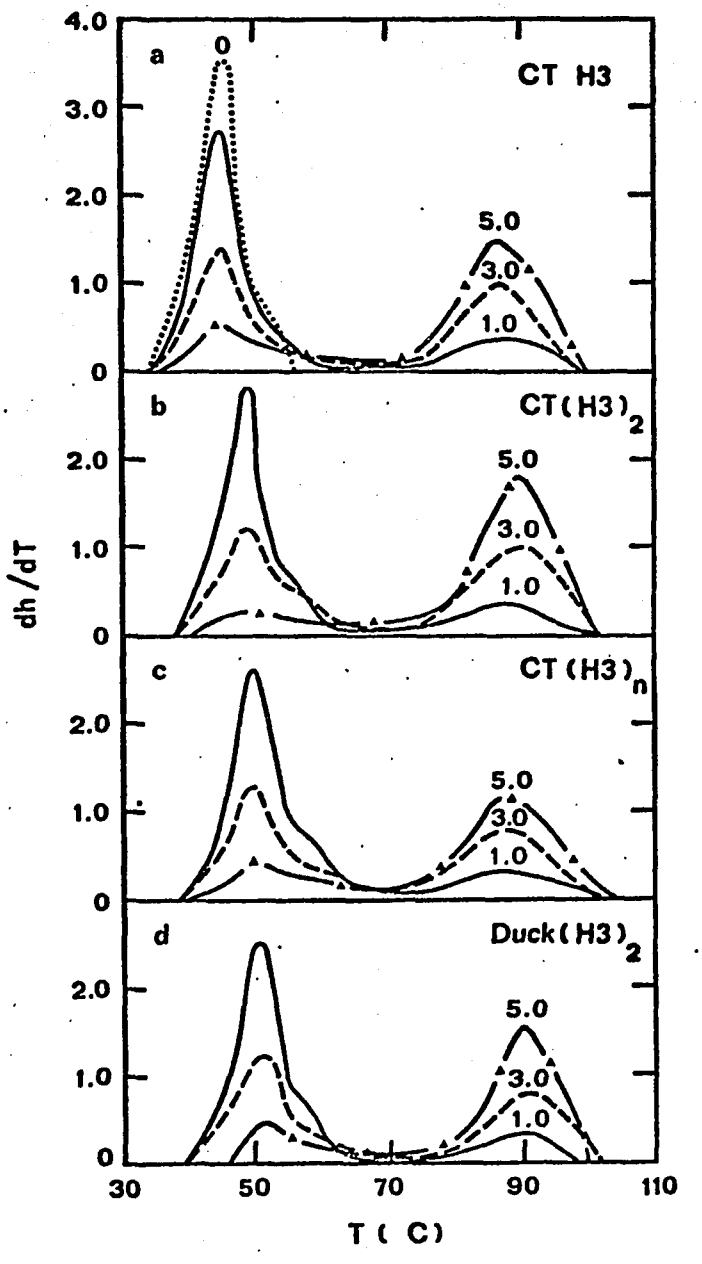


Fig. 30. Derivative melting profiles of various histone H3-DNA complexes prepared by direct mixing method. (a) Calf thymus histone H3 monomer-DNA complexes. (b) Calf thymus histone H3 dimer-DNA complexes. (c) Calf thymus histone H3 oligomers-DNA complexes. (d) Dutk histone H3 dimer-DNA complexes. The r values are 0 (····), 1.0 (——), 3.0 (---), and 5.0 (—▲—).

concomitant decrease in the amplitude at the T_m band. Except for minor variations, the melting curves of these complexes using various histone H3 are similar to one another.

Unlike melting curves from complexes formed by gradient dialysis with or without urea, the direct-mixed complexes have well separated and consistent melting curves which can be analyzed with the method described in Chapter II. Using Eq. (1),

$$r = \beta F = \beta \frac{A_{T_m'}}{A_T}$$

where $A_{T_m'}$ and A_T are respectively the melting areas under T_m' band and the whole melting cruve. A linear plot of r vs $A_{T_m'}/A_T$ yields β, the slope of the straight line. An example of such linear plot is shown in Figure 31 for the complex of DNA and histone H3 monomer. A β value of 6.7 amino acid residues per nucleotide is obtained. The β values of the DNA complexes using other histone H3 are given in TABLE VIII and are similar to one another. There is no significant difference between calf thymus and duck erythrocyte histone H3-DNA complexes.

CD spectra for calf thymus histone H3 monomer-DNA and dimer-DNA complexes are shown, respectively, in Figures 32a and 32b. Both histone H3 monomer and dimer have similar effects on DNA CD spectrum. There is a reduction in the amplitude and a very small red-shift of the CD band near 275 nm. The magnitude of these changes are directly proportional to the r value. There is also a big negative CD near 220 nm corresponding to the bound histones. In this case the histone H3 dimer-DNA complexes have a slightly bigger negative CD at 210 nm than the monomer-DNA complexes. The CD spectra of the directly mixed complexes (Figure 32) are closer to the CD spectrum of native chromatin than the reconstituted complexes

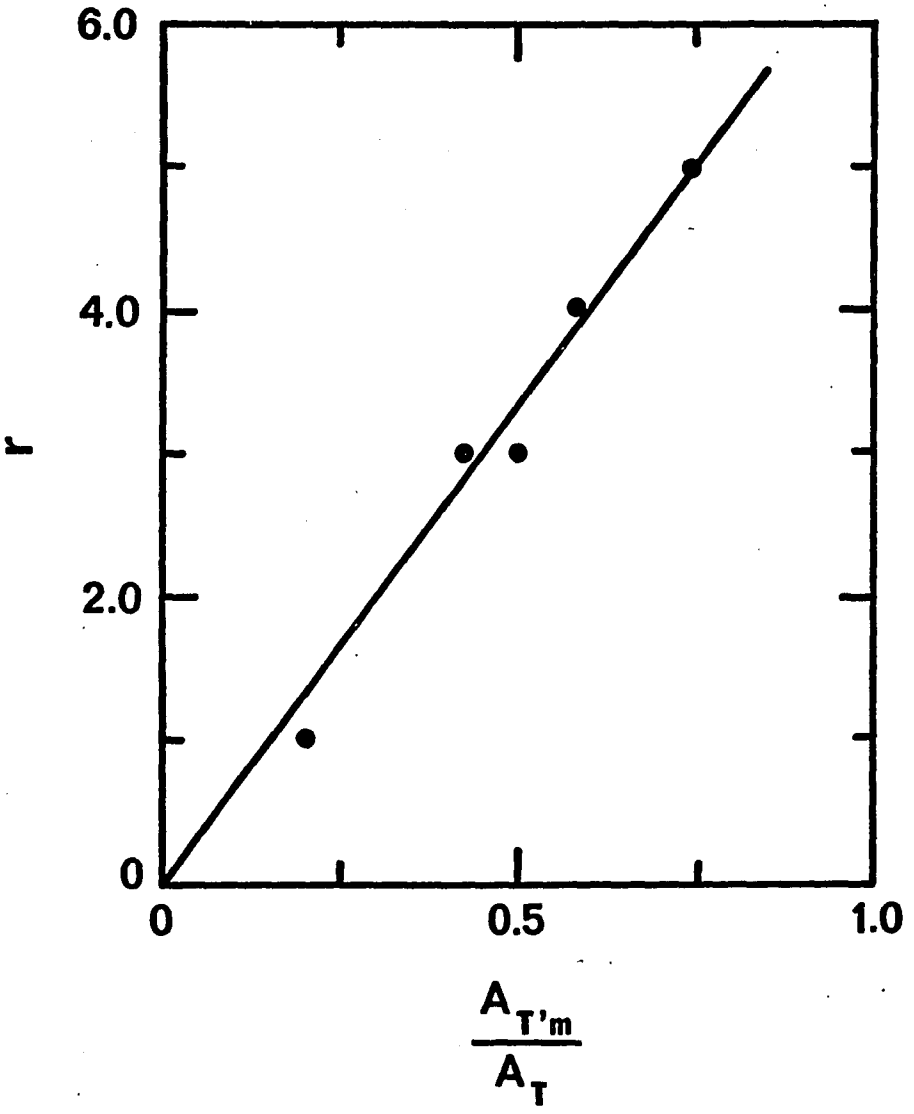


Fig. 31. Linear plot of equation (1) for calf thymus histone H3 monomer-DNA complexes prepared by direct mixing method. Melting areas for T_m and T_m' were obtained from derivative melting profile of histone H3-DNA complexes as shown in Fig. 30 (a).

TABLE VIII

The β Values of Various Histone H3-DNA Complexes Prepared by Direct Mixing

DNA Complexes	β
Calf thymus histone H3	6.5
Calf thymus histone $(H3)_2$	5.6
Calf thymus histone $(H3)_n$	5.8
Duck histone H3	6.4
Duck histone $(H3)_2$	5.9

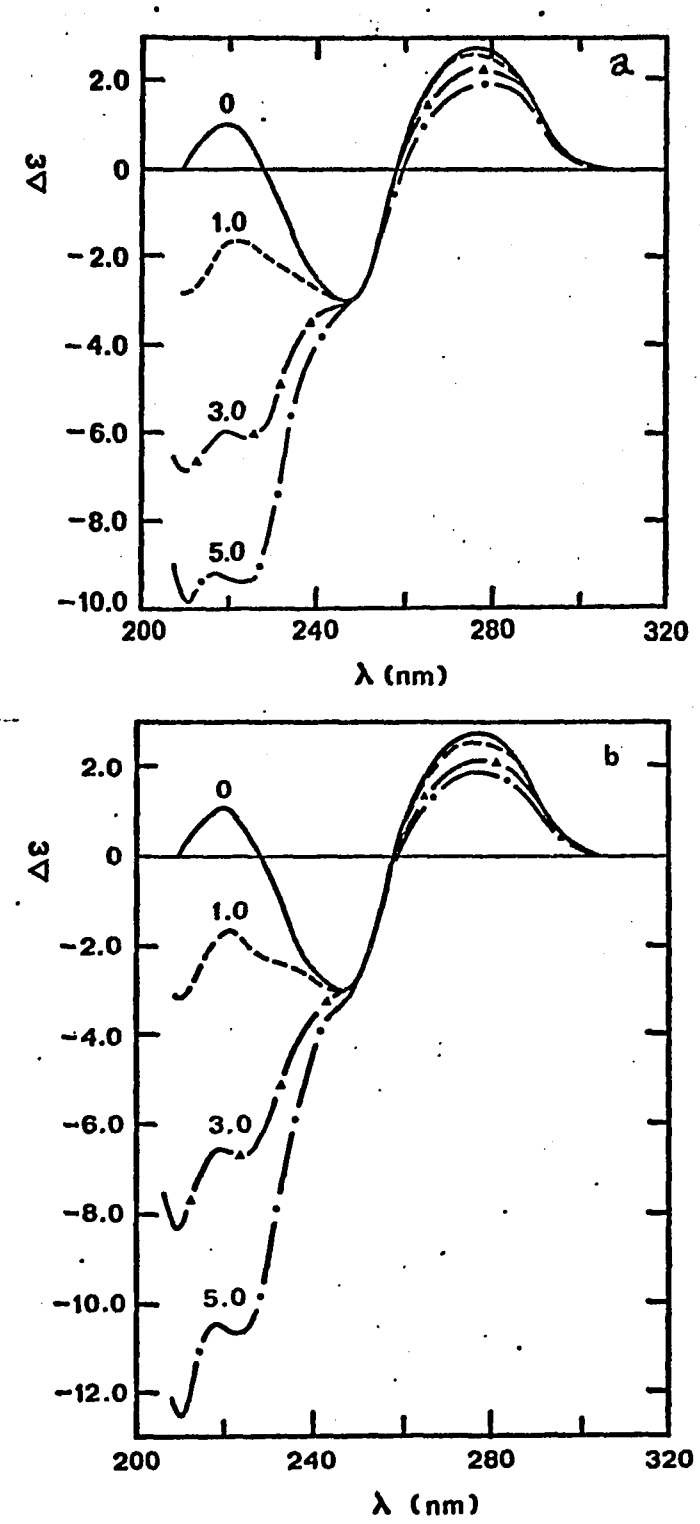


Fig. 32. CD spectra of calf thymus histone H3-DNA complexes prepared by direct mixing. (a) Histone H3 monomer-DNA complexes. (b) Histone H3 dimer-DNA complexes. The r values are 0 (——), 1.0 (---), 3.0 (—▲—), and 5.0 (— • —).

utilizing salt gradient dialysis with or without urea.

Since both the melting and CD results of these directly mixed complexes are well defined, the CD spectra for bound histone ($\Delta\epsilon_b^H$) and for DNA base pairs bound by histone ($\Delta\epsilon_b^D$) can be calculated using the following two equations:

$$\Delta\epsilon_m = F\ \Delta\epsilon_b^D + (1-F)\ \Delta\epsilon_f^D \qquad (8)$$

$$\Delta\epsilon_m = \Delta\epsilon_f^D + r\ \Delta\epsilon_b^H \qquad (10)$$

Eq. (8) is valid only for λ greater than 250 nm since CD in this region is contributed only by DNA base pairs either free ($\Delta\epsilon_f^D$) or histone-bound ($\Delta\epsilon_b^D$). F is the fraction of base pairs bound by histones in each complex as determined from the thermal denaturation results (Figure 31). The CD spectra of histone-bound base pairs, $\Delta\epsilon_b^D$, calculated from Eq. (8) for calf thymus histone H3-monomer-DNA complexes are shown in Figure 33a. $\Delta\epsilon_b^D$ is only two thirds as large as $\Delta\epsilon_f^D$, the CD of free DNA, and is slightly shifted to the red as judged from the crossover point. There is, however, no apparent shift in the peak position.

Below 250 nm, Eq. (10) is used for calculating the CD of bound histone ($\Delta\epsilon_b^H$) by assuming that the DNA CD in this region is not significantly changed and is small when compared to the CD contribution from histone. The calculated CD spectrum of bound histone H3 monomer ($\Delta\epsilon_b^H$) as shown in Figure 33b contains a negative peak at 220nm. The change in CD amplitude near 220 nm for this histone from a free state, $\Delta\epsilon_f^H$ (EDTA), to a complexed state, $\Delta\epsilon_b^H$, is small.

$\Delta\epsilon_b^H$ and $\Delta\epsilon_b^D$ for the various histone H3-DNA complexes were calculated as described for DNA-histone H3 monomer complex, and are shown in Figure 34. The CD effects of the different forms of histone H3 on DNA near 275 nm are similar to the monomer complex except that the amplitude varies

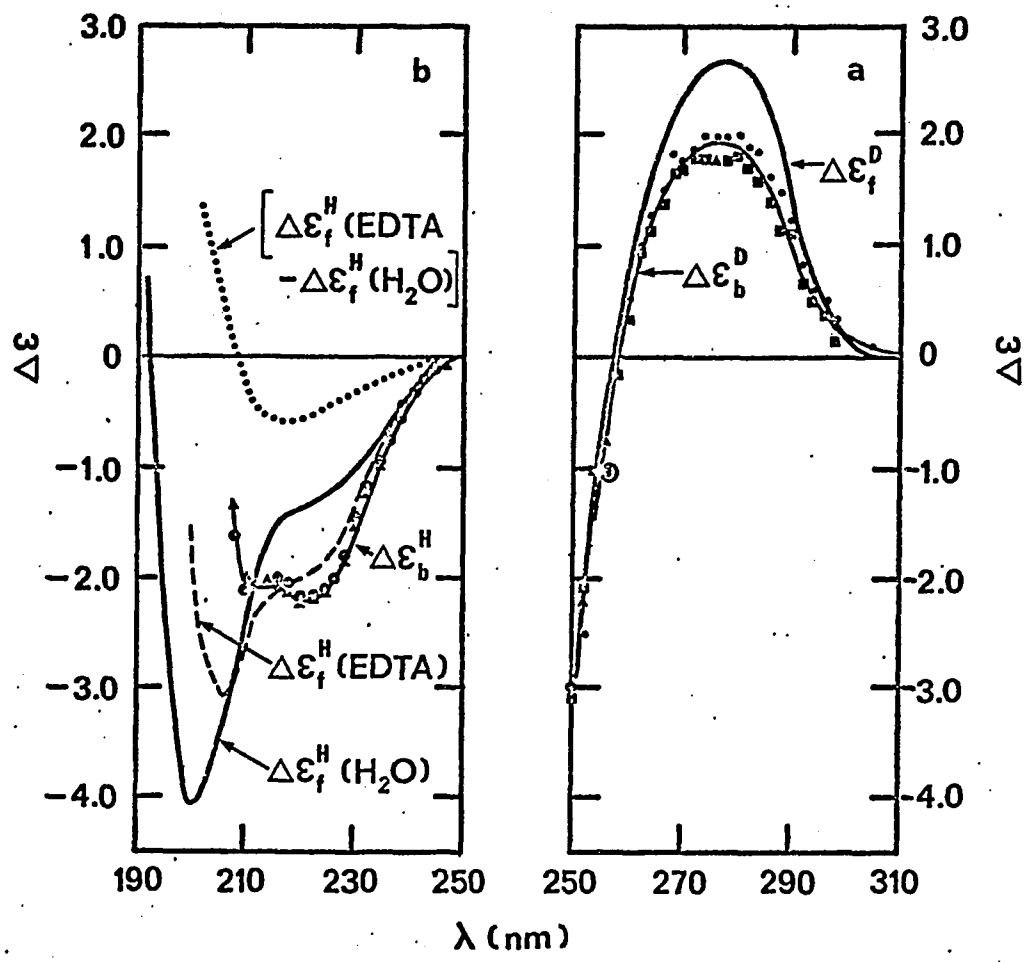


Fig. 33. Calculated CD of histone H3-DNA complexes prepared by direct mixing method. (a) $\Delta\epsilon_b^D$ of histone H3-bound DNA base pairs calculated from equation (8) using complexes with r= 1.0 (•), 3.0 (■), and 5.0 (▲). Also included is $\Delta\epsilon_f^D$ of free DNA. $\Delta\epsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of nucleotide. (b) $\Delta\epsilon_f^H$ of free histone H3 in water (——), in EDTA buffer and DTT (---) and the difference of these two spectra (···). $\Delta\epsilon_b^H$ of DNA-bound histone H3 calculated from equation (10) using complexes with r = 3.0 (•) and 5.0 (▲). $\Delta\epsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of amino acid residues of histones.

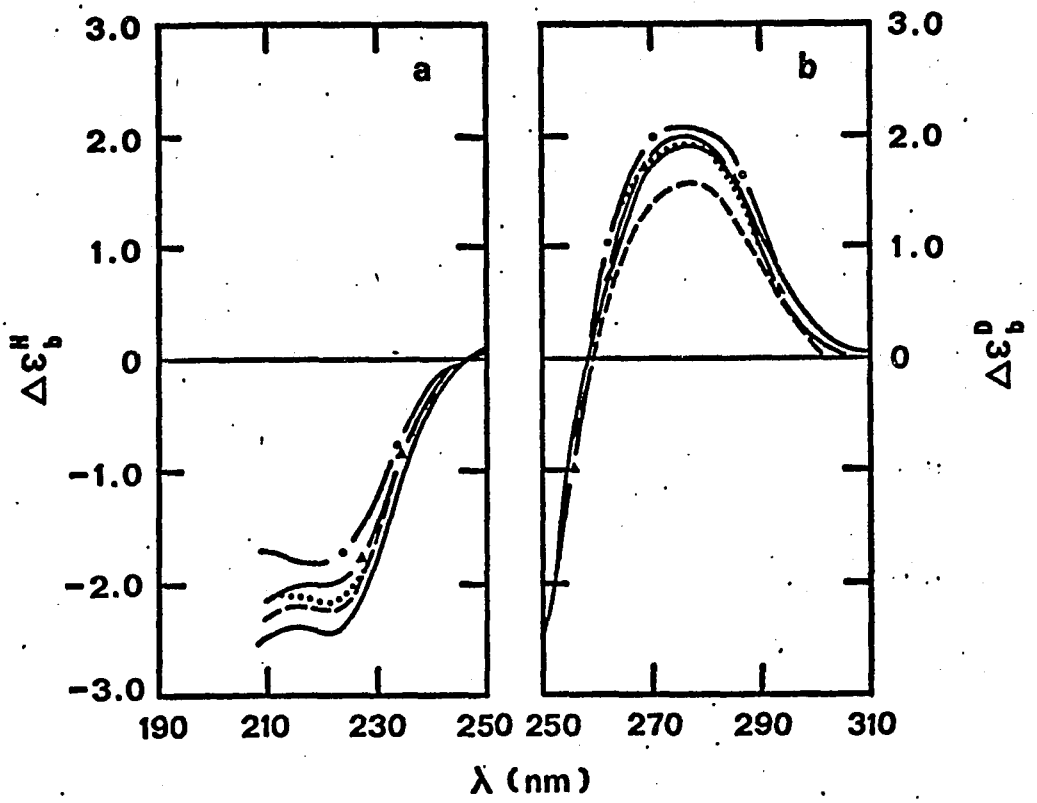


Fig. 34. Calculated CD of various histone H3-DNA complexes prepared by direct mixing method. (a) $\Delta\epsilon^{H}_{b}$ of various DNA-bound histone H3 calculated from equation (10). (b) $\Delta\epsilon^{D}_{b}$ of various histone H3-bound DNA base pairs calculated from equation (8). The various histone used for complexing are calf thymus histone H3 monomer (···), calf thymus histone H3 dimer (——), calf thymus histone H3 oligomers (---), duck histone H3 monomer (—·—), and duck histone H3 dimer (—▲—).

and follows the order of calf thymus histone H3 oligomers > calf thymus histone H3 dimer > calf thymus histone H3 monomer. The CD of bound histone, $\Delta\varepsilon_b^H$, of complexes with various forms of histone H3 is also similar to one another.

CD Spectra of Calf Thymus Histone H3 Monomer, Dimer, Oligomers and Duck Erythrocyte Histone H3 Monomer and Dimer

The qualitative and quantitative similarity, as measured by thermal denaturation and CD, of histone H3 monomer-, dimer-, oligomer-.DNA complexes prepared by direct mixing in EDTA buffer suggest that there are similar secondary structures in the complexes with various forms of histone H3. In order to examine their secondary structures in free state the CD spectra of calf thymus histone H3 monomer, dimer, oligomers, duck erythrocyte histone H3 monomer, dimer in water (Figure 35a) and in 2.5×10^{-4}M EDTA buffer (Figure 35b) were measured. All histone spectrum in water has a major negative peak near 200 nm and a shoulder near 220 nm, suggesting the presence of random coil structure. Although the various histone spectra in water are qualitatively similar to one another, quantitatively they are still different. This variation could indicate the presence of different amounts of secondary structures in histones even in water. If one utilized magnitude of the negative CD at 215 nm or 222 nm as an indicator of the content of ordered secondary structures, calf thymus histone H3 monomer with two cysteine residues should have more ordered structures than duck histone H3 monomer with one cysteine. For both calf thymus and duck erythrocyte histone H3, the dimers have more ordered structures than the corresponding monomers. This is reasonable since the dimeric histone molecules with disulfide bonds should be closer enough to form intermolecular hydrogen bonds such as those of β-sheet. The smaller amplitude of oligomer in water

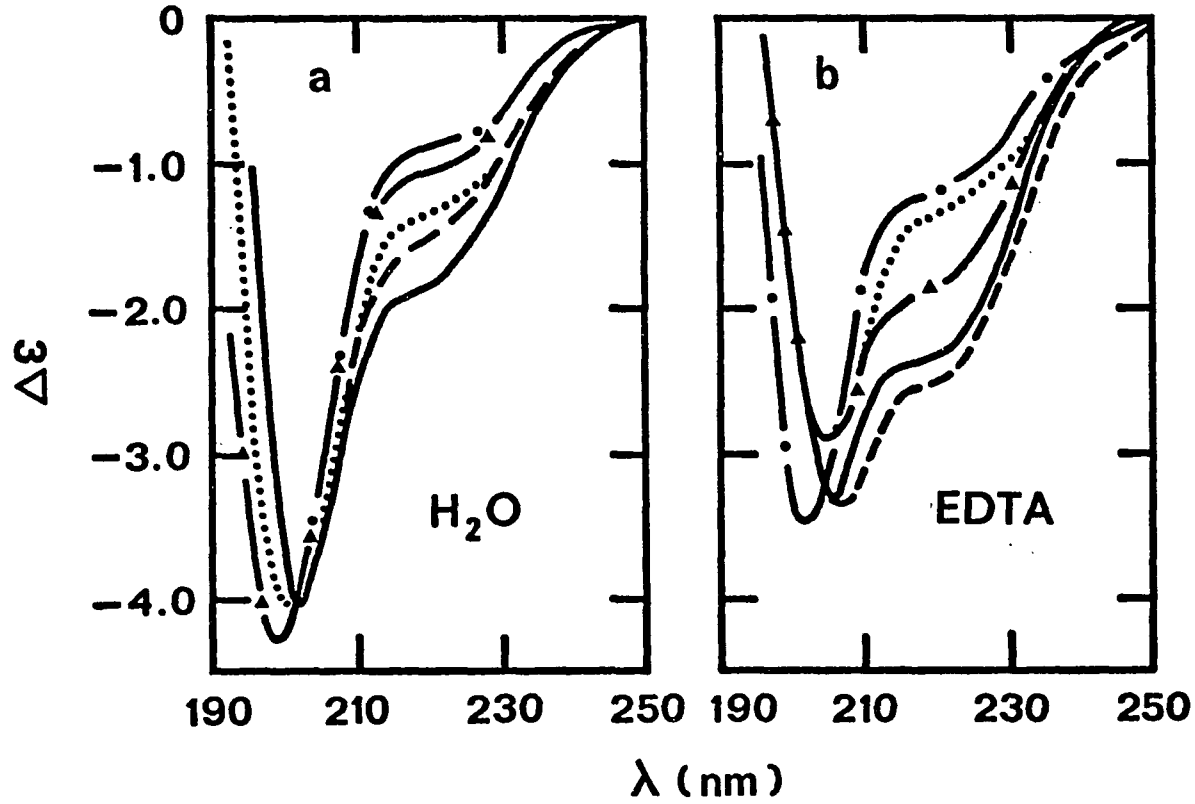


Fig. 35. CD spectra of various histones H3 in water (a) and in 2.5×10^{-4} M EDTA (b). The various histone used are calf thymus histone H3 monomer (• • •), dimer (——), oligomers (---) and duck histone H3 monomer (—•—) and dimer (—▲—). $\Delta\epsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of amino acid residues of histones.

than dimer could be a result of steric hindrance in hydrogen bond formation or due to charge repulsion.

The CD spectra of various histones in EDTA buffer have a reduced amplitude below 210 nm and an increased one around 220 nm, when compared to the spectra in water. These results indicate that each histone has more ordered structure in EDTA buffer than in water. The facilitation of ordered structure in EDTA buffer may be due to charge neutralization and stabilization of hydrogen bonds. There is a pronounced difference in CD spectra between histone H3 monomer and dimer from both calf thymus and duck erythrocyte in EDTA buffer. The difference in negativity is most distinct around 220 nm, and is more severe in calf thymus than in duck erythrocyte. Although calf thymus histone H3 oligomer in water has a smaller negative CD near 220 nm than the dimer, this order has been reversed in EDTA buffer. These results can be consistently explained as that the formation of disulfide bond facilitates the formation of intermolecular hydrogen bonding by holding the two histone molecules close to each other.

DISCUSSION

Sodium chloride gradient dialysis with urea (Bekhor et al., 1969; Huang and Huang, 1969) has been used frequently for reconstituting chromatin or nucleohistones. Characteristic melting properties of chromatin have been successfully reproduced by this method for reconstituted nucleohistones, using histone (H2A + H2B) and less successfully using H2B alone (Leffak et al., 1974). As shown in this chapter reconstituted complexes using histone H3 possess melting and CD properties very different from those of native chromatin. For the histone H3-DNA

complexes, the method of direct mixing in low ionic strength is better than NaCl gradient dialysis without urea which is still better than NaCl gradient dialysis with urea, when melting and CD properties of native chromatin are used as criteria for successful complex formation. This order is quite surprising because it is generally assumed that NaCl gradient dialysis with urea is the best method for reconstitution.

The rational for using NaCl gradient dialysis with urea is that urea is expected to reduce histone-histone interaction. In the case of histone H4, urea was shown to reduce histone aggregation (Li and Isenberg, 1972). The current subunit model of histone-DNA interaction, however, required the formation of histone-histone interaction prior to binding with DNA (Van Holde et al., 1974; Li, 1975). Since interaction among histones could possibly be destroyed or reduced by urea, the binding of histone H3 to DNA may become undefined and hence the instability of a good histone H3-DNA complex to form in urea.

The quantitative similarity in melting results of DNA complexes prepared by direct mixing using histone H3 monomer, dimer, oligomers from calf thymus and histone H3 monomer and dimer from duck erythrocytes (Figure 30) suggest that only the more basic regions of histone H3 directly interact with DNA phosphates. The less basic regions in histone H3, where the content in -SH group varies and the formation of disulfide bond occurrs, either stay outside of DNA or are only loosely bound to the opposite side of the same DNA segment already bound by the more basic regions. This interpretation is plausible because the T'_m at 88-90° is closer to the highest melting band (about 82°) in chromatin which has been interpreted as due to the binding of the more basic regions of histones (Li and Bonner, 1971; Li et al., 1973). In addition, the efficiency of covering DNA by

histone H3 in the present complexes (β = 6.7) is roughly one half that of chromatin [β = 3.0 to 3.5 amino acid residues per nucleotide in histone-bound regions of chromatin (Li, 1973; Li et al., 1973)] where both halves of histones are directly associated with adjacent fragments of DNA.

CHAPTER VI

INTERACTION BETWEEN HISTONE H4 AND DNA, AND BETWEEN HISTONE (H3 + H4) AND DNA

Histone aggregation in neutral salt solutions was a problem in the early days of histone identification and fractionation. However, it has become a key evidence in the recent chromosomal subunit theory (Kornberg and Thomas, 1974; Van Holde et al., 1974; Olins and Olins, 1974) when it was realized that histone interactions are specific (D'Anna and Isenberg, 1974b). As wes discussed in Chapter I, the major interacting histones are the two Arg-rich histones (histones H3 and H4) which form dimeric or tetrameric subunits. The two slightly Lys-rich histones (histones H2A and H2B) also form dimeric subunit (Kelley, 1973). Interaction between histone H4 and H2A have also been reported (D'Anna and Isenberg, 1974b).

Evidences for the interaction of histones have been obtained mostly by chemical cross-linking reaction either directly in chromatin (Martinson and McCarty, 1975; Hyde and Walker, 1975) or with histone complexes extracted from chromatin (Kornberg and Thomas, 1974). Recently, D'Anna and Isenberg (1974c) have found that histone H3 and H4 when mixed equimolarly

in 10 mM sodium phosphate buffer at pH 7.0 formed a complex with molecular weight corresponding to a tetrameric structure.

In this chapter, DNA and histone (H3 + H4) tetramer complexes were studied by thermal denaturation and circular dichroism methods. Histone H4-DNA complexes and histone (H3 + H4)-DNA complexes prepared by the methods of gradient dialysis with urea, gradient dialysis without urea and direct mixing were also studied.

RESULTS

A. Histone H4-DNA and Histone (H3 + H4)-DNA Complexes Prepared by Gradient Dialysis Without Urea and by Direct Mixing

Thermal denaturation profiles of histone H4-DNA and histone (H3 + H4)-DNA complexes prepared by gradient dialysis without urea are shown in Figure 36. Thermal denaturation profiles for these complexes prepared by the direct mixing method are shown in Figure 37. For histone (H3 + H4)-DNA complexes, an equimolar of histone H3 monomer and histone H4 were mixed before complexing with DNA. As was found for histone H3-DNA complexes (Chapter V), direct mixing method yields complexes with sharp and well melting curves, a melting band at 47° (T_m) for free DNA and another one at 86° for histone-bound DNA. The amplitude of the melting band at T_m' increases with higher r value while the amplitude of T_m band decreases concomitantly. Although histone H4-DNA and histone (H3 + H4)-DNA complexes prepared by gradient dialysis without urea also gave T_m' at about 90°, the amplitude is not well proportion to the r value and for histone H4-DNA complexes, the T_m melting band was shifted slightly to higher temperature. This phenomenon is undoubtedly due to aggregation of histone H4 in NaCl without urea since copious white precipitates were found with the histone H4-DNA complex.

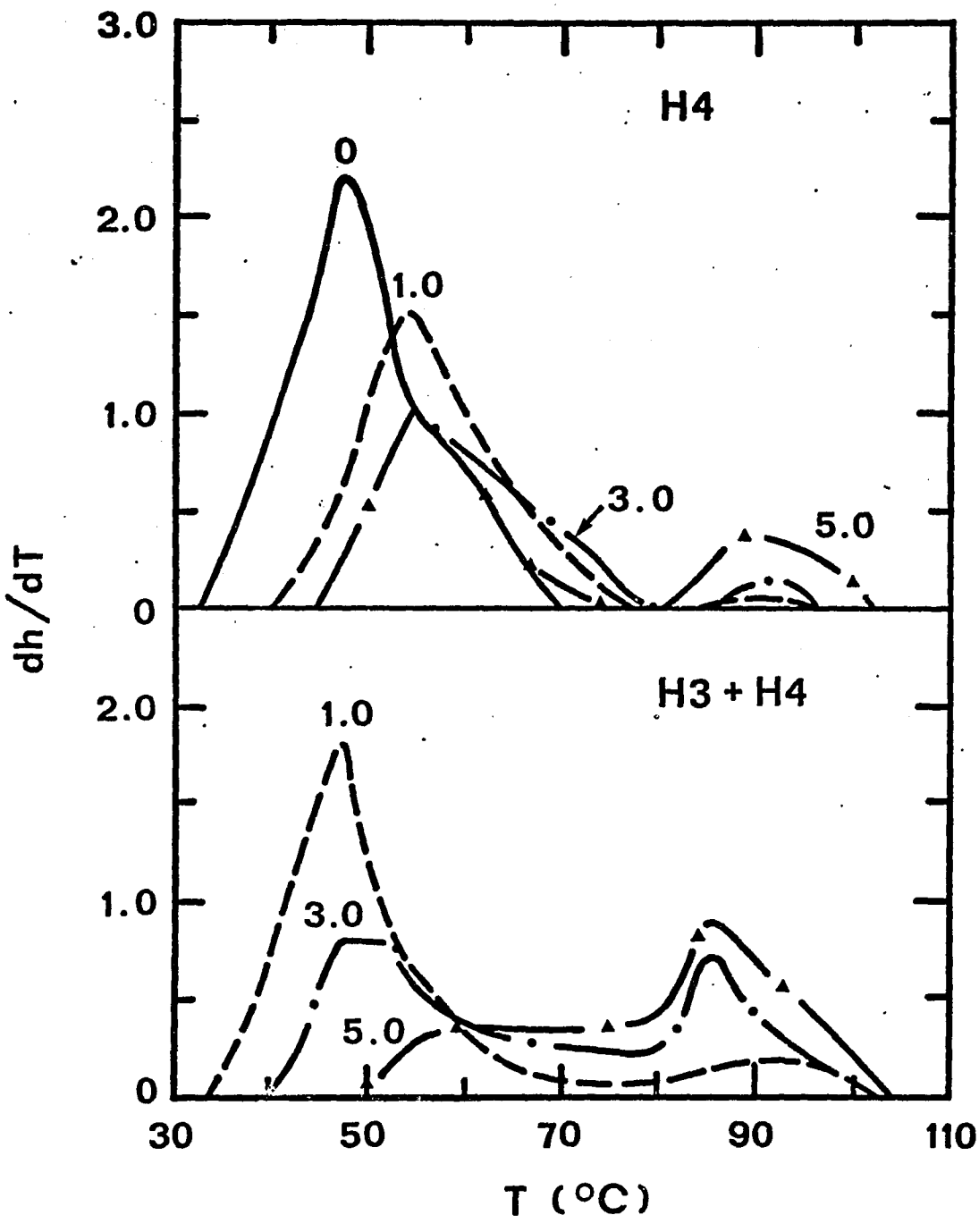


Fig. 36. Derivative melting profiles of histone H4-DNA (Upper panel) and histone (H3+H4)-DNA complexes (Lower Panel) prepared by gradient dialysis without urea. The r values are 1.0 (---), 3.0 (-·-), and 5.0 (—▲—). Histone (H3 + H4)-DNA complexes were prepared by mixing equimolar of each histone before complexing with DNA.

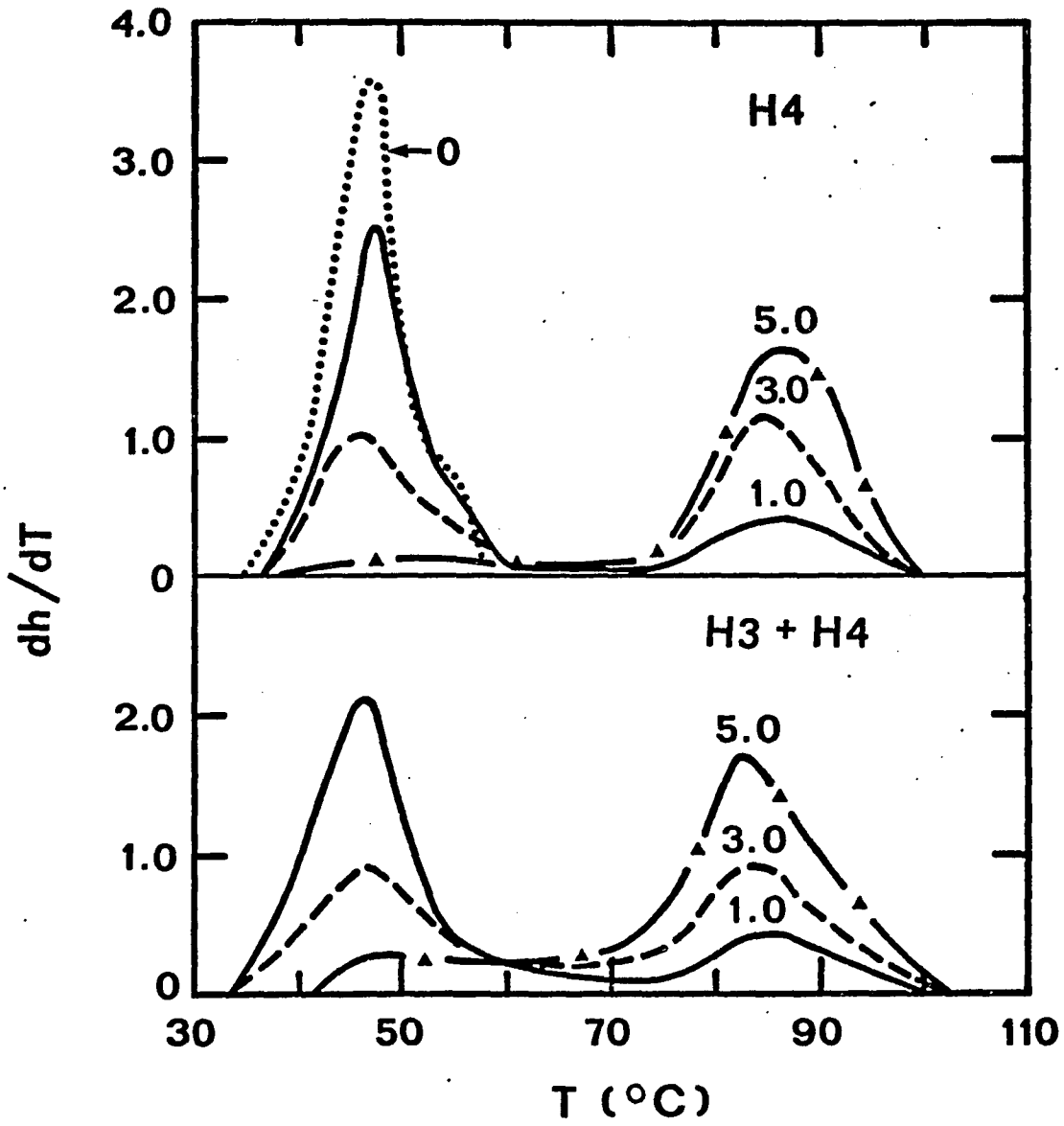


Fig. 37. Derivative melting profiles of histone H4-DNA (Upper panel) and Histone (H3 + H4)-DNA complexes (Lower panel) prepared by direct mixing. The r values are 0 (····), 1.0 (——), 3.0 (---), and 5.0 (—▲—).

B. Histone H4-DNA and Histone (H3 + H4)-DNA Complexes Prepared by Gradient Dialysis With Urea

Histone H4-DNA and histone (H3 + H4)-DNA complexes prepared by gradient dialysis with urea have rather well defined biphasic melting curves (Figure 38). This result is different from that of histone H3-DNA complex prepared by the same method where no defined melting bands were found (Chapter V). Both histone H4-DNA and histone (H3 + H4)-DNA complexes have T'_m at about 90^o and the amplitude of this melting band increases with increasing r value. Beside the melting bands at T_m and T'_m there is an additional broad melting band around 55^o for both complexes. The amplitude of this melting band, however, decreases with increasing r value. Both complexes have strong light scattering in the final preparation especially for those with higher r values. This indicates that aggregation could also occur in these complexes.

C. Preparation of Histone (H3 + H4) Tetramer

Histone (H3 + H4) tetrameric complex was prepared by the method of D'Anna and Isenberg (1974c). Equimolar of histone H4 and histone H3 monomer or in case of histone H3 dimer, at half the concentration, were mixed in water. The ionic environment of the medium was then increased to 10 mM sodium phosphate, pH 7.0 by the addition of concentrated phosphate buffer (100 mM). The histone solution was then let stand in ice for at least 10 min. before using it for complexing with DNA.

To form DNA-histone tetramer complex, the histone tetramer solution was slowly added to DNA solution also in 10 mM phosphate with constant stirring. Portions of the DNA- histone tetramer complexes were first dialyzed overnight against 0.1 M NaCl, 10 mM Tris-Cl, pH 8.0 at 4^o and

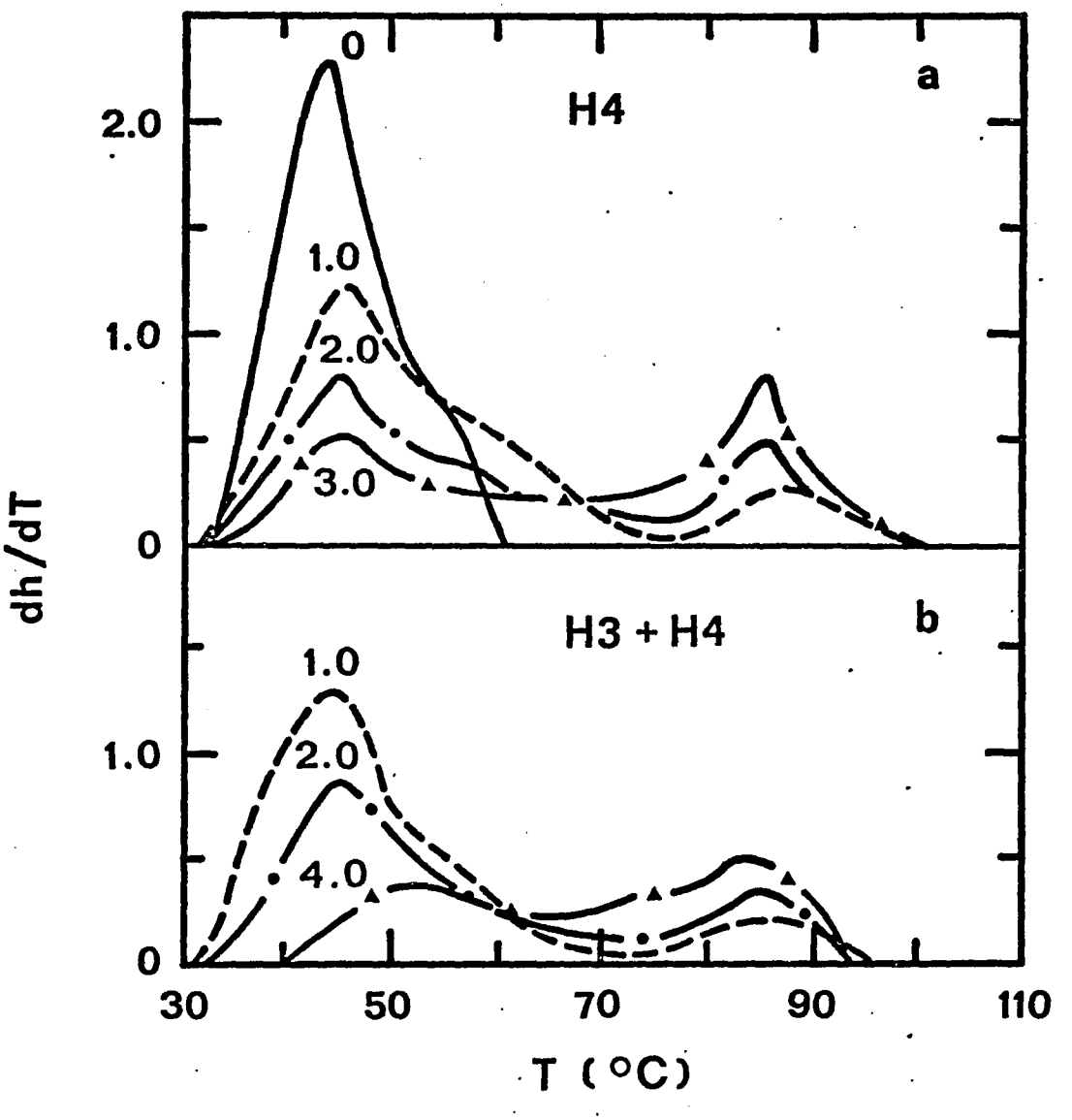


Fig. 38. Derivative melting profiles of histone H4-DNA and histone (H3 + H4)-DNA complexes prepared by gradient dialysis with urea. (a) Histone H4-DNA complexes with r values of 0 (——), 1.0 (---), 2.0 (—•—), and 3.0 (—▲—). (b) Histone (H3 + H4)-DNA complexes with r values of 1.0 (---), 2.0 (—•—), and 4.0 (—▲—).

subsequently dialyzed with several changes against 2.5 x 10^{-4}M EDTA, pH 8.0. Without the intermediate step of dialysis against 0.1M NaCl buffer, prolong dialysis against EDTA buffer (5 to 6 days) is required to remove phosphate completely.

D. Histone H3-, H4-, and (H3 + H4) Tetramer-DNA Complexes Prepared by Direct Mixing in Phosphate Buffer

The phenomenon of histone H3 and H4 forming a complex with a discrete size in salt solution is in contrast to that of histone H3 or H4 alone in the same salt solution. In latter case random aggregation of histone H3 or H4 could occur and ultimately precipitate out of the solution at high salt.

If histone (H3 + H4) tetramer, prepared in 10 mM phosphate buffer, is complexed with DNA, also in 10 mM sodium phosphate buffer, thermal denaturation of these complexes show a melting band of free DNA at 72° (T_m), and another melting band of histone-bound DNA at 87° (T'_m) (Figure 39a). With increasing r value the amplitute of T'_m band increases while that of T_m band decreases. Thermal denaturation properties of these complexes were also studied after dialysis into EDTA buffer (Figure 39b). It was found that, for unknown reason a prolong dialysis (5 to 6 days) against EDTA buffer with several changes of the buffer was required to lower down the T_m from 72° of free DNA in phosphate buffer to about 50° in EDTA buffer. Figure 39b shows that, unlike the shift of T_m, the melting band at T'_m is reduced only by 2° (87°to 85°) after the complexes were dialyzed to EDTA buffer with lower ionic strength. This result indicates that the phosphate lattice in histone-bound regions is highly neutralized by histone (H3 + H4) tetramer through ionic binding of the basic amino acid residues. Furthermore, these complexes in EDTA buffer

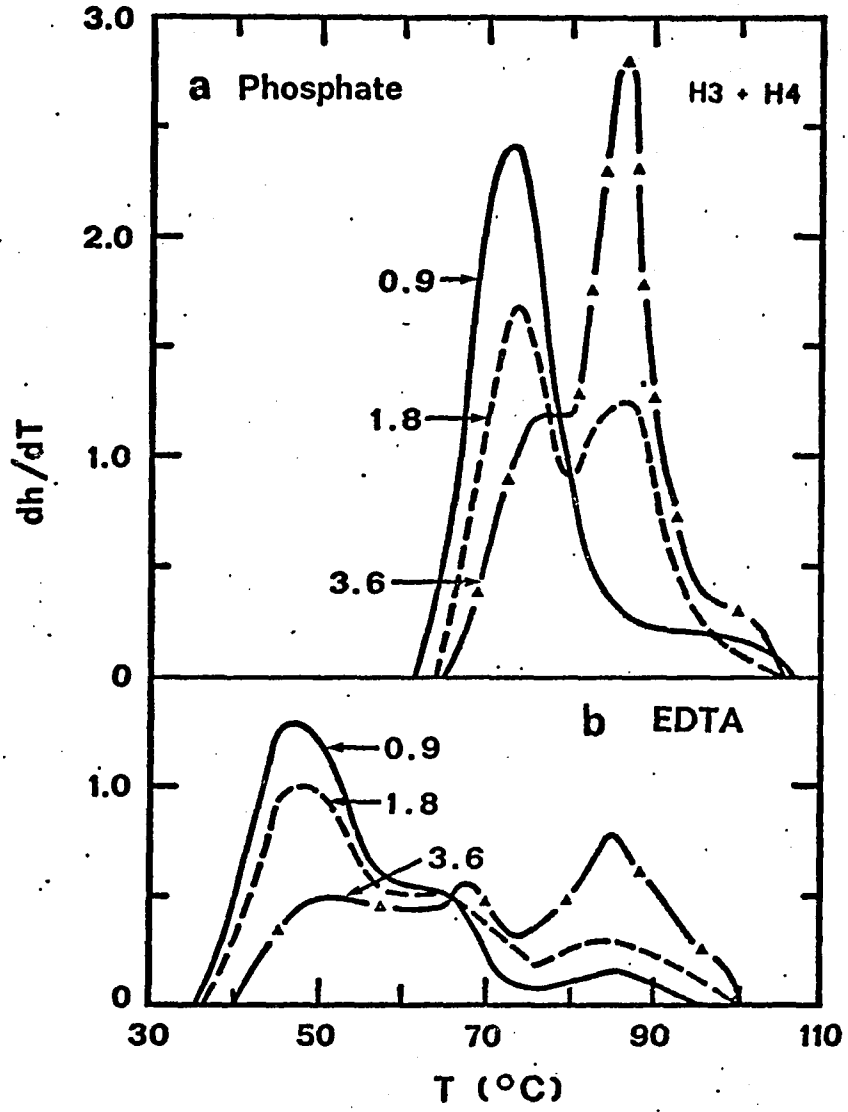


Fig. 39. Derivative melting profiles of histone (H3 + H4)-tetramer-DNA complexes. (a) Histone (H3 + H4) tetramer-DNA complexes were prepared by direct mixing in 10 mM sodium phosphate buffer, pH 7.0. (b) An aliquot of complexes was dialyzed for 5 days with several changes of 2.5×10^{-4} M EDTA, pH 8.0 and melted in EDTA buffer. The r values of complexes are 0.9 (——), 1.8 (---), and 3.6 (—▲—).

show a broad melting band around 65°.

If the complexes in phosphate buffer were first dialyzed against 0.1 M NaCl in 0.01 M Tris buffer and subsequently dialyzed against EDTA buffer, the melting profiles shown in Figure 39b are readily reproduced after one day of dialysis. Using this method, DNA complexes with histone H3, histone H4, histone (H3 + H4) tetramer and histone (H3 dimer + H4) tetramer were prepared and their thermal denaturation properties were studied (Figure 40). DNA complexes with histone H3 and histone H4 alone show typical melting profiles with a T_m of 50°, a T'_m of 85° and a small broad melting between 65° to 75°.

DNA-histone complexes using (H3 + H4) tetramer or (H3 dimer + H4) tetramer show some significant increase in the melting near 65° (Figures 40c and d) . The amplitude of this band increases with r value until r = 3.0 after that, further addition of histone causes a decrease of this band with a concomitant increases in the amplitude at T'_m band (85°). It is important to note that complexes of histone (H3 dimer + H4) tetramer with DNA have melting properties similar to those of histone (H3 + H4) tetramer-DNA complexes. This may indicate that histone H3 dimer is capable of forming same tetrameric structure with histone H4 as is the case for histone H3 monomer.

In contrast to the cloudy suspension found for histone (H3 + H4)-DNA complexes prepared by gradient dialysis with or without urea, preformation of histone (H3 + H4) tetramer or histone (H3 dimer + H4) tetramer, followed by complexing with DNA results in a clear solution. These results suggest that histone H3 and H4 (or H3 dimer and H4) form natural subunits and these subunits can form good complexes with DNA

Fig. 40. Derivative melting profiles of histone-DNA complexes prepared in phosphate buffer. Histone H4-DNA (a), histone H3-DNA (b), histone (H3 + H4) tetramer=DNA (c) and histone (H3 dimer + H4) tetramer-DNA complexes (d) were prepared by direct mixing in 10 mM phosphate buffer, pH 7.0. The complexes were dialyzed overnight at 4°C against 0.1 M NaCl, 0.01 M Tris-HCl, pH 8.0 and then against several changes of 2.5×10^{-4} M EDTA. The r values of the complexes are 0 (···), 1.0 (——), 2.0 (---), 3.0 (-·-), and 5.0 (—▲—). All complexes were melted in 2.5×10^{-4} M EDTA.

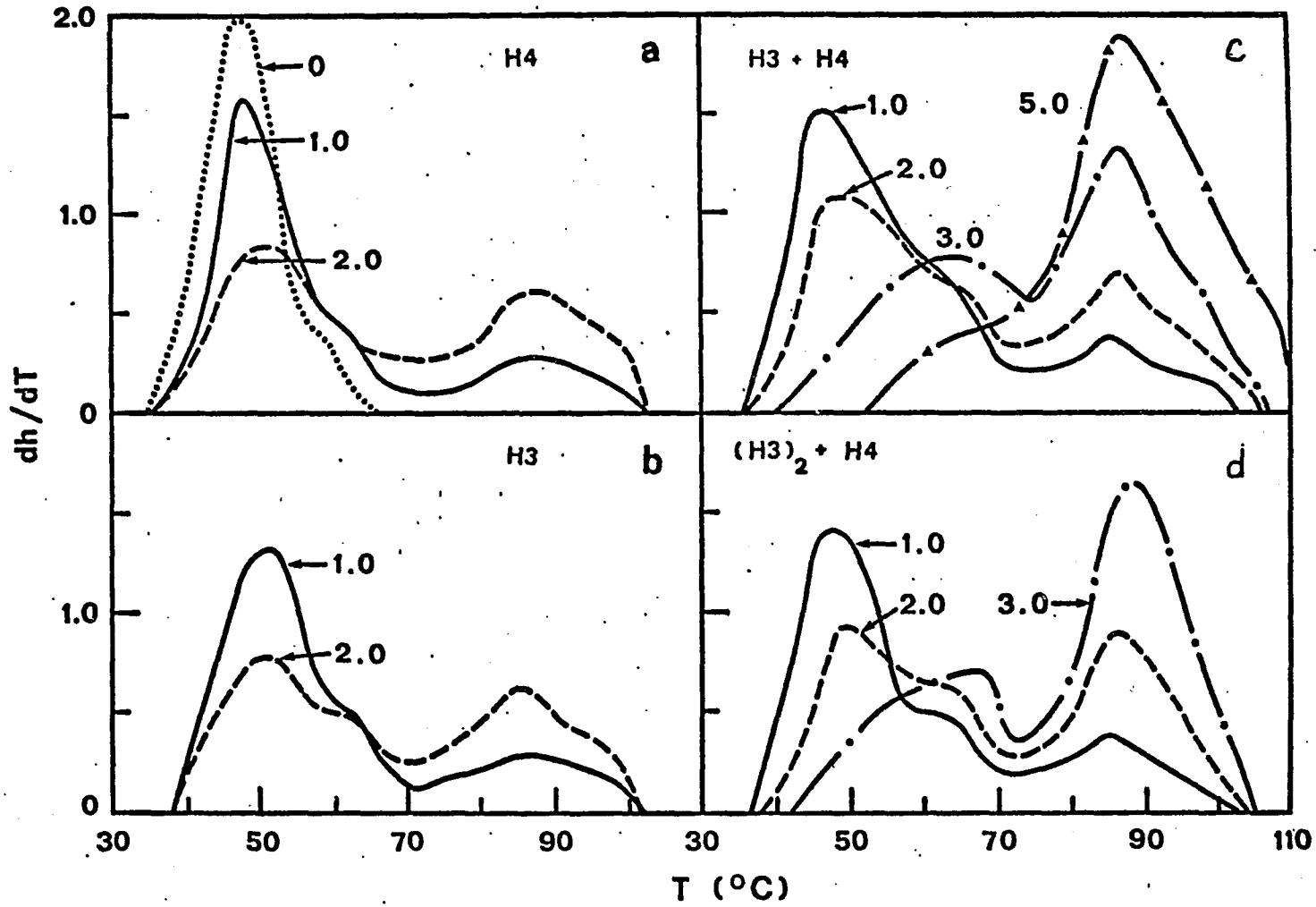

2.0
1.0
0
dh/dT
H4
a
0
1.0
2.0
H3 + H4
c
1.0
5.0
2.0
3.0
H3
b
1.0
2.0
(H3)2 + H4
d
1.0
2.0
3.0
30
50
70
90
30
50
70
90
110
T (°C)

Fig. 40

without severe non-specific aggregation which might have occurred when histone H3, H4, or H3 + H4 was used in NaCl with or without urea.

E. Analysis of Thermal Denaturation Results

As discussed in Chapter II, for well defined melting curves the β values (amino acid residue/nucleotide in histone-bound regions) can be obtained from the slope of a linear plot of r vs $A_{T_m'}/A_T$. Figure 41 illustrates the results of histone (H3 + H4)-DNA complexes prepared by direct mixing in EDTA buffer. A β value of 6.0 was obtained. This value is comparable to that (β = 6.7) obtained for histone H3 monomer-DNA complexes and histone H4-DNA complexes (β = 5.2) prepared by a similar method.

As shown in Figure 40, those complexes using histone H3, histone H4, histone (H3 + H4) tetramer and (H3 dimer + H4) tetramer have two histone bound melting bands, the major one at 90° and the minor one at 65°. In these cases, $A_{T_m'}$ were calculated as the sum of melting area under the 90° and 65° melting bands. Data for these types of complexes were also plotted in Figure 41 and a β value of 2.8 was obtained for the two tetramer-DNA complexes. This value is about half that obtained for histone H3 monomer-DNA and histone (H3 + H4)-DNA complexes prepared by the direct mixing method and is about equal to that obtained for native chromatin, where β is 3.0 - 3.5 (Li, 1973). Histone H3- and histone H4-DNA Complexes prepared by the same method yield a β value of 4.0.

F. Circular Dichroism of Complexes Prepared by Gradient Dialysis With and Without Urea and by Direct Mixing

Circular dichroism spectra of histone H4-DNA and histone (H3 + H4)-DNA complexes prepared by gradient dialysis with urea, without urea, and

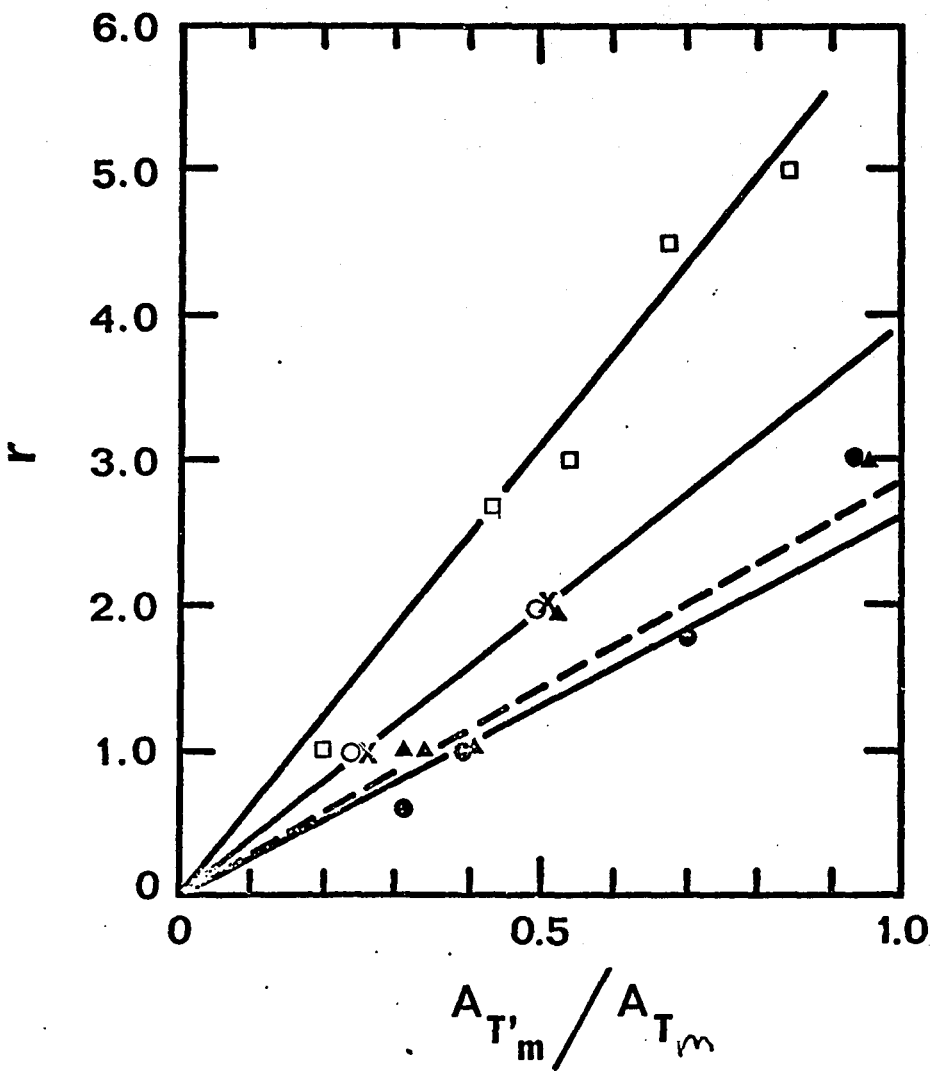


Fig. 41. Linear plots of equation (1) for histone-DNA complexes prepared in phosphate buffer. Melting areas for T_m and T'_m were obtained from derivative melting profiles of histone-DNA complexes described in Fig. 40. Data for histone H4-DNA (– o –), histone H3-DNA (– x –), histone (H3 + H4) tetramer-DNA (---▲---), and histone (H3 dimer + H4) tetramer-DNA (– ● –) complexes were calculated. Included is also data from melting profiles of histone (H3 + H4)-DNA complexes prepared by direct mixing in EDTA (–□–).

direct mixing are depicted in Figures 42, 43 and 44 respectively.

Histone H4-DNA complexes prepared by gradient dialysis with urea show a reduced amplitude for the CD band near 275 nm with increasing r values (Figure 42a). The peak at 275 nm and the cross-over point for the CD band are also strongly red-shifted with higher r values. The same phenomonon is observed for histone H3-DNA complex prepared by the same method (Chapter V). The presence of both histone H3 and H4 during complex formation by this method reduces the CD effect on red-shift of both the peak and the cross-over point and on amplitude (Figure 42b). The negative CD near 220-230 nm is attributable to the bound histones. The amplitude of this peak is increased with increasing r values. However, the amplitude is much smaller than that of chromatin.

Figure 43 shows the CD spectra of histone-DNA complexes prepared by gradient dialysis without urea. Histone H4-DNA complexes prepared this way show no red-shift of either the peak or the cross-over point in the CD band near 270 nm, although the amplitude of this peak is still depressed with higher r values. This result is different from histone H4-DNA complex prepared by gradient dialysis with urea. The CD band near 220 nm has a smaller amplitude than that of a complex prepared by gradient dialysis with urea (Figure 42). Similarly, histone (H3 + H4)-DNA complexes prepared by gradient dialysis without urea also have a smaller red-shift of their 275 nm CD band and a larger amplitude at 220 nm than complexes prepared by gradient dialysis with urea.

CD spectra of DNA-histone complexes prepared by direct mixing are shown in Figure 44. Histone H4-DNA complex prepared by this method shows no red-shift in their 275 nm CD band. As is the case in histone H3 monomer-DNA complex, more secondary structure of histones is preserved by direct

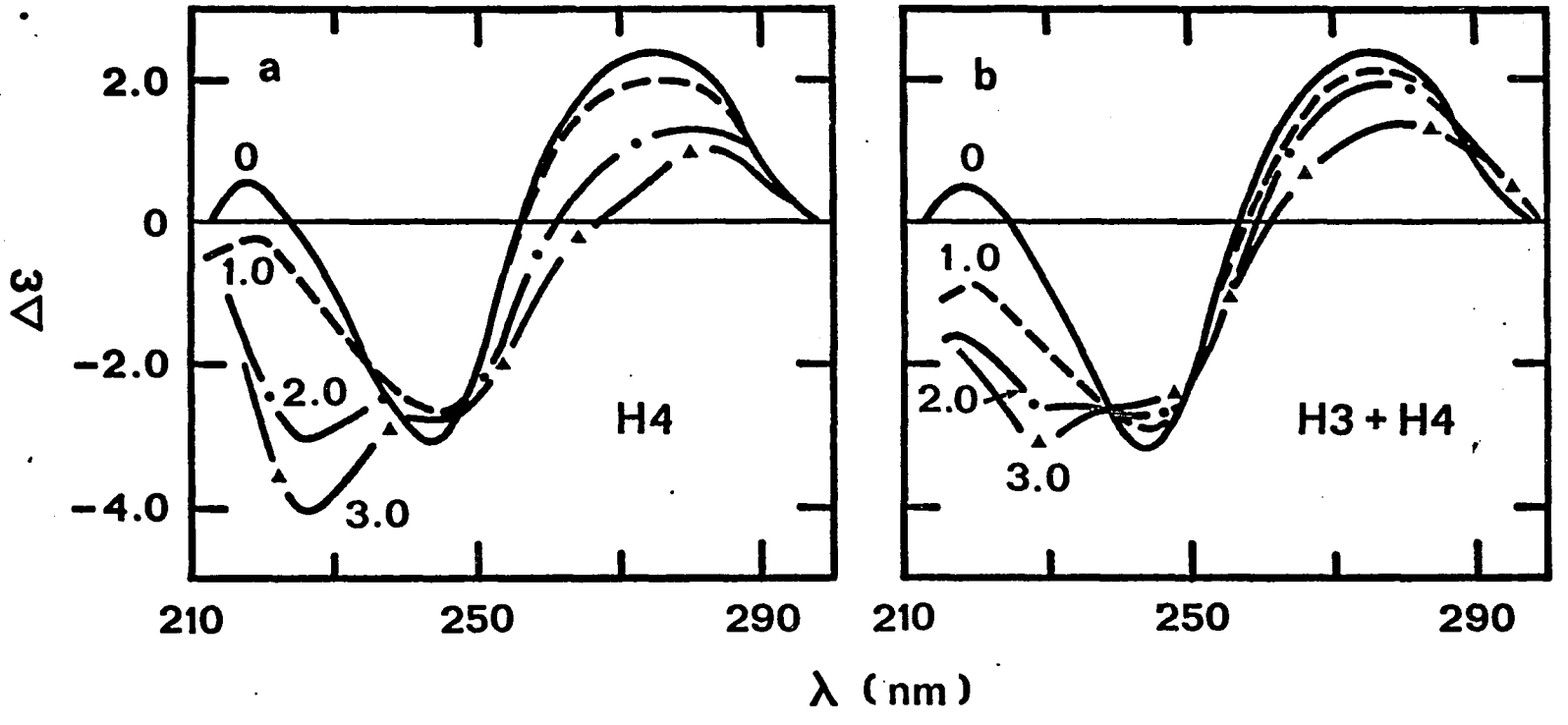


Fig. 42. CD spectra of histone H4-DNA (a) and histone (H3 + H4)-DNA complexes (b) prepared by gradient dialysis with urea. The r values are 0 (——), 1.0 (- - -), 2.0 (—·—), and 3.0 (—▲—). Histone (H3 + H4)-DNA complexes were prepared by mixing equimolar of each histone before complexing with DNA.

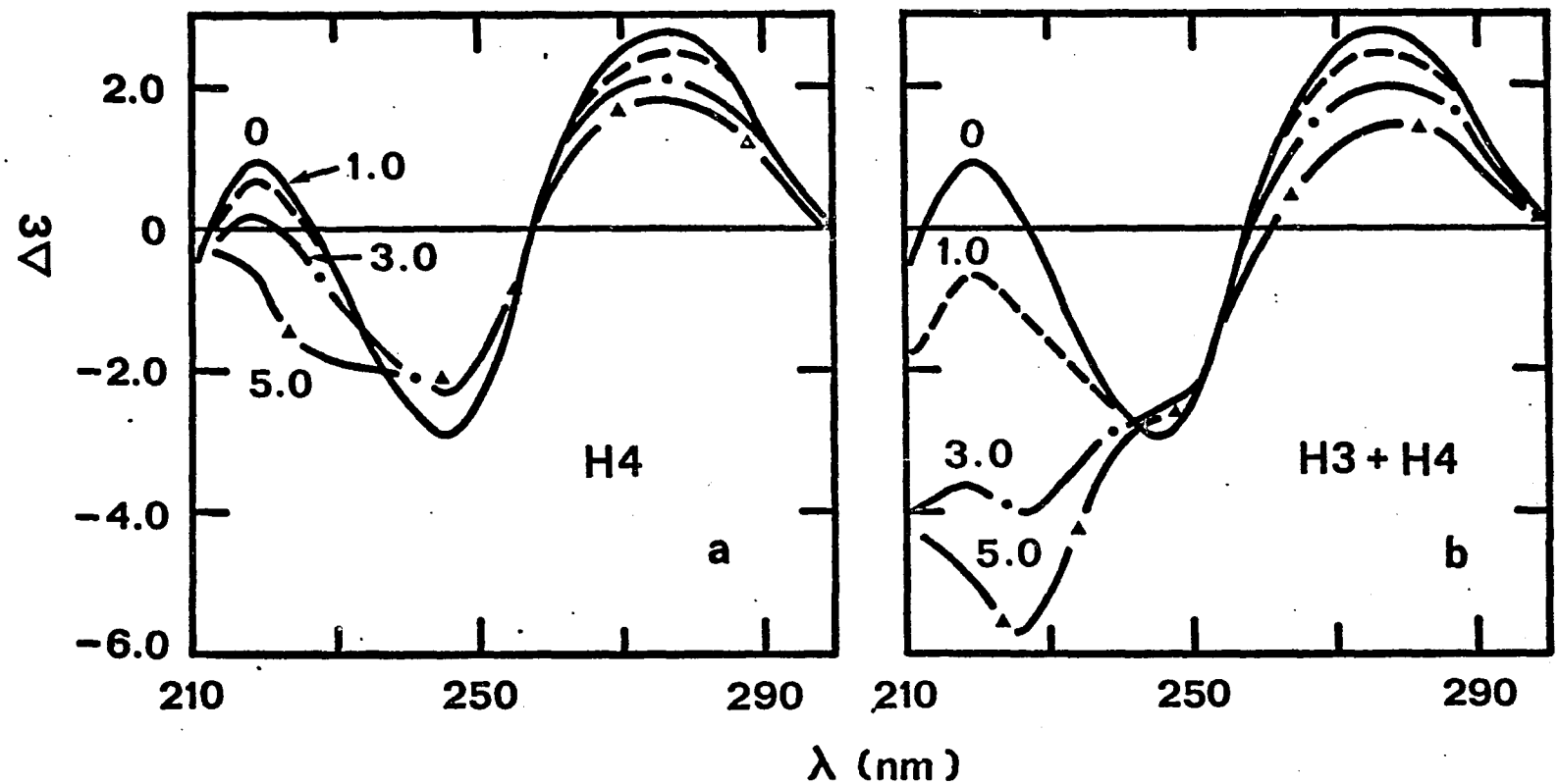


Fig. 43. CD spectra of histone H4-DNA (a) and histone (H3 + H4)-DNA complexes (b) prepared by gradient dialysis without urea. The r values are 0 (——), 1.0 (---), 3.0 (—·—), and 5.0 (-▲-).

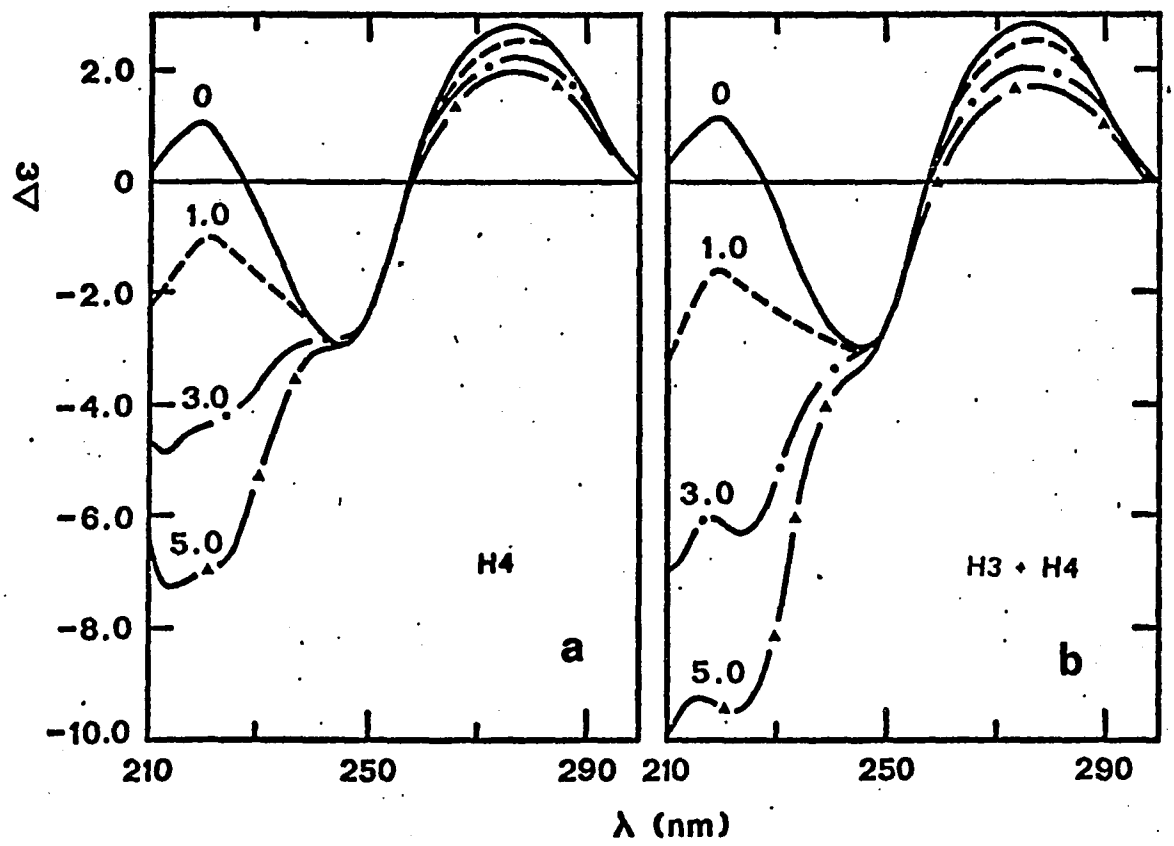


Fig. 44. CD spectra of histone H4-DNA (a) and histone (H3 + H4)-DNA complexes (b) prepared by direct mixing. The r values are 0 (——), 1.0 (---), 3.0 (-•-), and 5.0 (-▲-).

mixing method as shown by the large negative CD near 220nm. An even more negative CD at 220 nm is observed for histone (H3 + H4)-DNA complex. The increase in amplitude at 220 nm is proportional to the increase in r value. Histone (H3 + H4)-DNA complexes show a slight red-shift for their 275 nm CD band.

Comparing the three methods of complex formation, the greatest red-shift and reduction of the amplitudes in DNA CD near 275 nm is found for complexes prepared by gradient dialysis with urea (Figure 42). The greatest degree of histone secondary structure as measured by the negative CD at 220 nm is found for complexes prepared by the direct mixing method (Figure 44). In all three methods, the presence of both histone H3 and histone H4 in the DNA complex has generated CD spectra different from those of complexes when histone H3 or H4 is used.

G. Circular Dichroism of Histone (H3 + H4) Tetramer-DNA Complexes

The CD spectra of histone (H3 + H4) tetramer-DNA complexes are shown in Figure 45. The CD spectra of these complexes are very similar to those of histone (H3 + H4)-DNA complexes prepared by direct mixing in EDTA buffer (Figure 44), despite the great difference found in their thermal denaturation results (See Figures 37 and 40).

H. Calculation of CD Parameters

The CD spectra for bound histone ($\Delta\epsilon_b^H$) and for DNA base pairs bound by histones ($\Delta\epsilon_b^D$) were calculated for histone H4-DNA complexes prepared by the direct mixing method in EDTA buffer (Figure 46) and for histone (H3 + H4) tetramer-DNA and histone (H3 dimer + H4) tetramer-DNA (Figure 47). All three different histone-DNA complexes have $\Delta\epsilon_b^D$ about 3/4 as large as

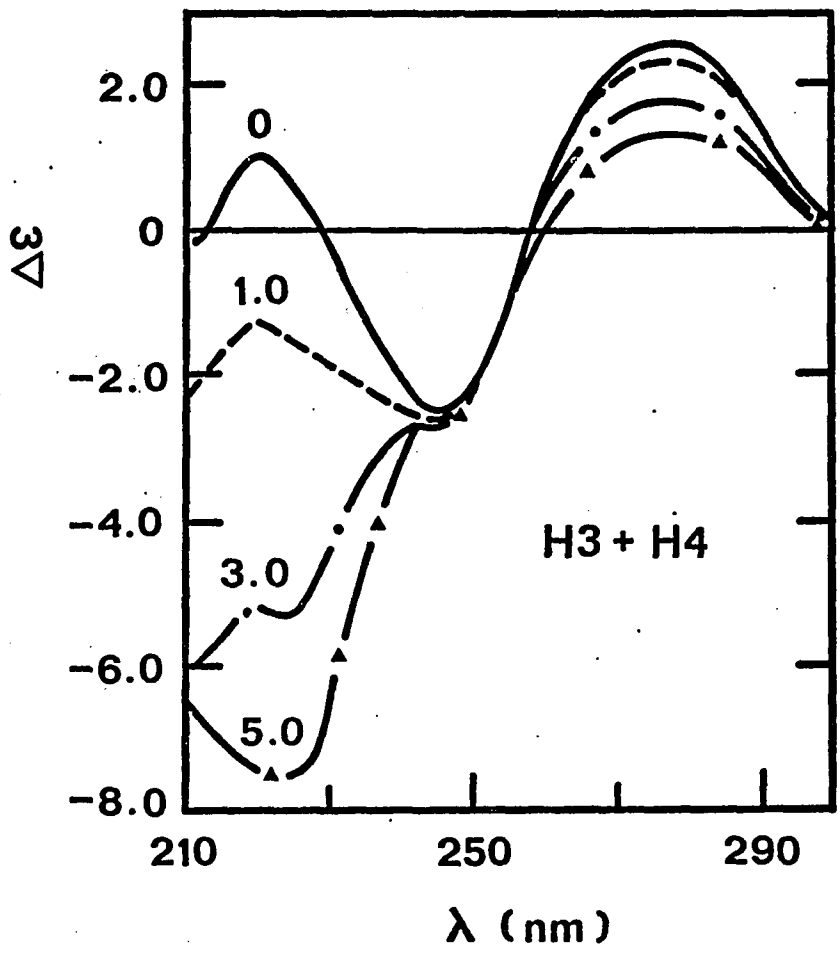


Fig. 45. CD spectra of histone (H3 + H4) tetramer-DNA complexes. The complexes were prepared by direct mixing in 10 mM phosphate buffer, pH 7.0 then dialyzed overnight at 4°C against 0.1 M NaCl, 0.01 M Tris-HCl, pH 8.0 and then against several changes of 2.5×10^{-4} M EDTA. The r values are 0 (——), 1.0 (---), 3.0 (—·—), and 5.0 (—▲—).

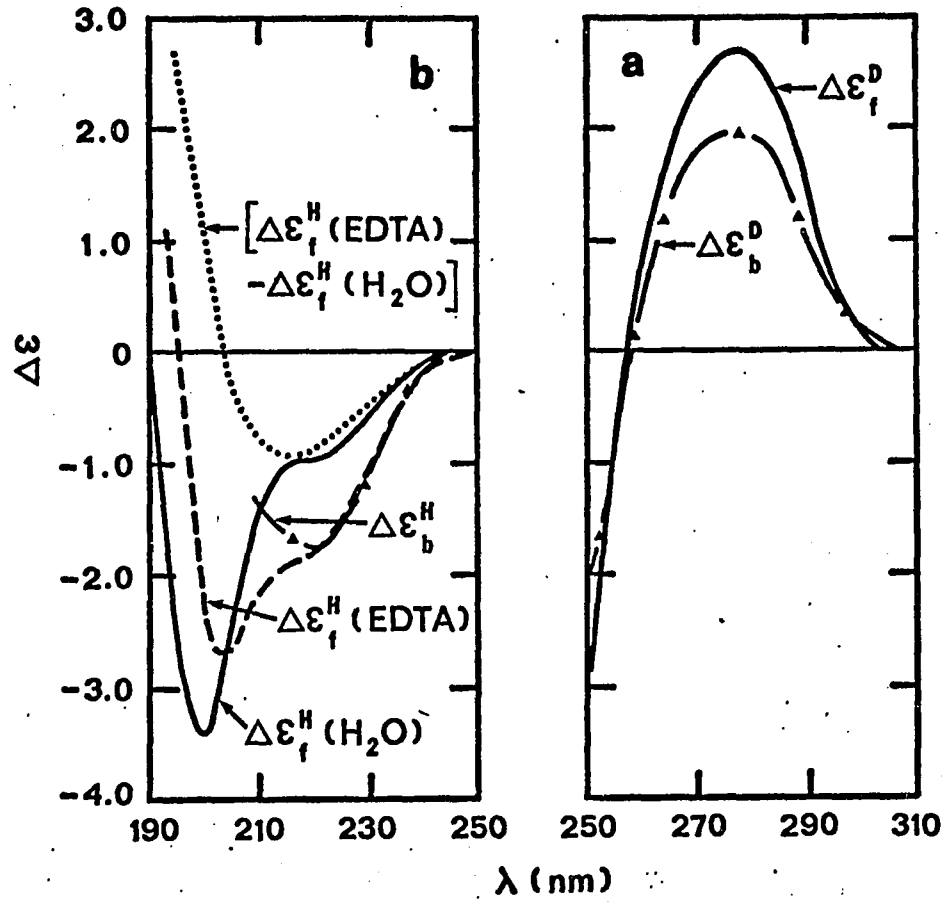


Fig. 46. Calculated CD of histone H4-DNA complexes prepared by direct mixing method. (a) $\Delta\varepsilon_b^D$ of histone H4-bound DNA base pairs were calculated from equation (8). Also included is $\Delta\varepsilon_f^D$ of free DNA. $\Delta\varepsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of nucleotide. (b) $\Delta\varepsilon_f^H$ of free histone H4 in H_2O (——), in EDTA buffer (---) were determined and the difference of these two spectra (····) calculated. $\Delta\varepsilon_b^H$ (—▲—) of DNA-bound histone H4 were calculated from equation (10). $\Delta\varepsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of amino acid residues of histones.

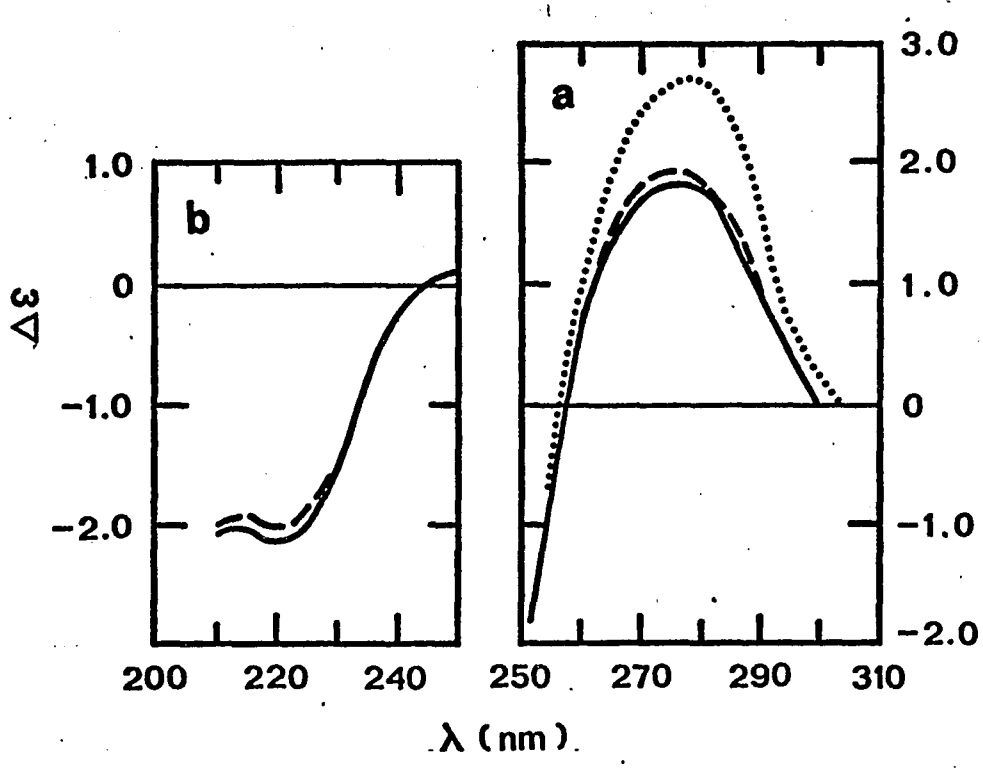


Fig. 47. Calculated CD of histone (H3 + H4) tetramer-DNA complexes prepared by direct mixing in phosphate buffer. (a) $\Delta\varepsilon_b$ of histone (H3 + H4) tetramer-bound DNA (——) and histone (H3 dimer + H4) tetramer-bound DNA (---) base pairs were calculated from equation (8). Also included is $\Delta\varepsilon_f$ of free DNA (···). $\Delta\varepsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of nucleotide. (b) $\Delta\varepsilon_b$ of DNA-bound histone (H3 + H4) tetramer (—) and histone (H3 dimer + H4) tetramer (---) were calculated from equation (10). $\Delta\varepsilon$ is in $M^{-1}cm^{-1}$ where M is moles/liter of amino acid residues of histones.

that of $\Delta\varepsilon_f^D$ of free DNA. There is no red shift of the 275 nm peak position and there is only a very slight shift for the crossover point (Figures 46 and 47).

The calculated CD spectrum of bound histone H4 ($\Delta\varepsilon_b^H$) is shown in Figure 46. There is only a very small difference in CD amplitude near 220 nm between the bound ($\Delta\varepsilon_b^H$) and the free ($\Delta\varepsilon_f^H$) state of histone H4 in EDTA buffer. The calculated CD spectra of bound histone (H3 + H4) tetramer and bound histone (H3 dimer + H4) tetramer are nearly identical to each other.

I. CD Spectrum of Histone (H3 + H4) Tetramer

The CD spectra of histone (H3 + H4) in water, in EDTA and in 10 mM phosphate buffer, where they form the tetrameric structure, were shown in Figure 48. The spectrum in water has a major negative peak at 200 nm and a shoulder at 220 nm. This CD spectrum is decreased below 210 nm and increased around 220 nm when the histones are in EDTA buffer. The CD spectrum of histone (H3 + H4) tetramer in 10 mM phosphate becomes more negative at both 220 and 205 nm than when the histone are in EDTA buffer. This difference is clearly depicted in the difference spectrum $[\Delta\varepsilon_f^H(PO_4) - \Delta\varepsilon_f^H(H_2O)]$ and $[\Delta\varepsilon_f^H(EDTA) - \Delta\varepsilon_f^H(H_2O)]$ shown in Figure 48, and indicates that an increase in secondary structure, possibly α-helixes, in histone (H3 + H4) tetramer in 10 mM phosphate buffer.

DISCUSSION

Histone H4-DNA complexes formed by gradient dialysis with urea show well-defined biphasic melting profiles, while histone H3-DNA complexes formed by the same method show only broad melting curves (Chapter V).

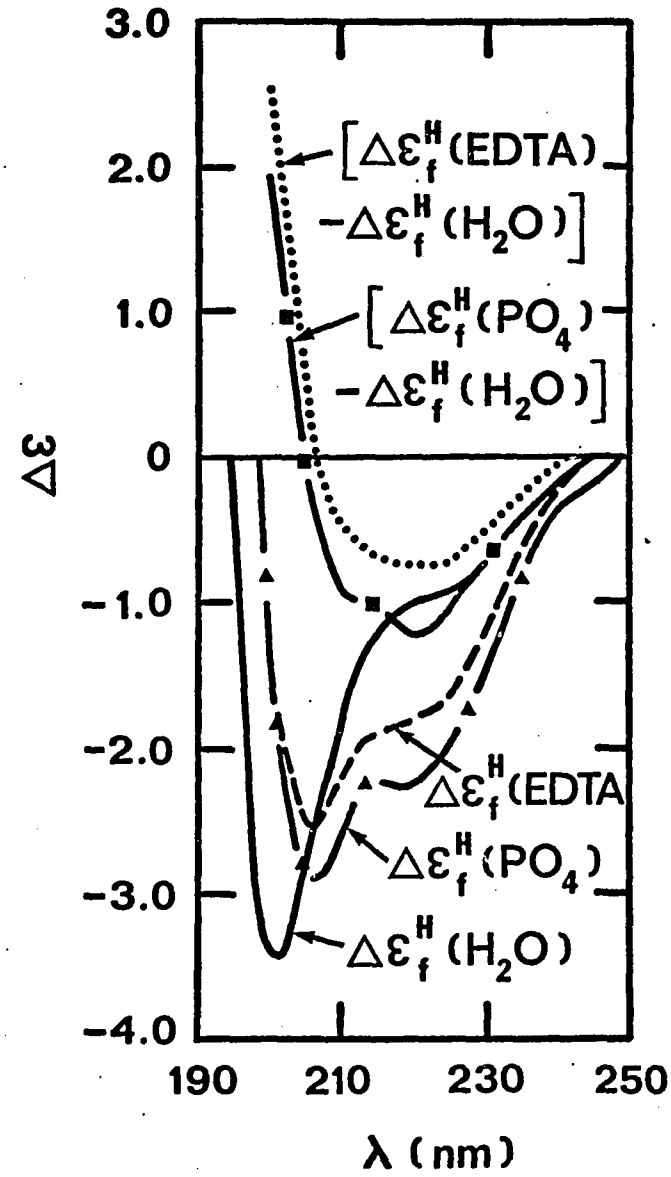


Fig. 48. CD spectra of histone (H3 + H4) tetramer. $\Delta\varepsilon_f^H$ of free histone (H3 + H4) tetramer in water (——), in EDTA (---), and in phosphate buffer (—▲—) were measured and the difference spectra of $[\Delta\varepsilon_f^H(\text{EDTA}) - \Delta\varepsilon_f^H(H_2O)]$ (····) and $[\Delta\varepsilon_f^H(PO_4) - \Delta\varepsilon_f^H(H_2O)]$ (—■—) were determined.

Complexes of DNA and histone (H3 + H4) prepared by a similar method also have biphasic melting curves. These results indicate that histone H3 alone may not form a well-defined complex in NaCl gradient with urea. The presence of histone H4 may facilitate the formation of subunit of histone H3 and H4 before they bind the DNA. CD results seems to support this interpretation. In both histone H4-DNA and histone H3-DNA complexes, both the peak of the 275 nm CD band and the crossover point are greatly red-shifted. The magnitute of these shifts are reduced in histone (H3 + H4)-DNA complexes which is similar to that found in chromatin (Shih and Fasman, 1970).

Both histone H3 and H4 have more secondary structures in EDTA buffer than in water as measured by the amplitu e of the CD band near 220 nm. The difference CD spectrum shows a negative peak near 215 nm, (Figures 33 and 46) suggesting an increased amount of the secondary structures, most likely β-sheet, in EDTA buffer. For histone (H3 + H4) tetramer in 10 mM phosphate buffer, the difference CD spectrum shows a major negative peak at 220 nm and aminor one at 210 nm (Figure 48) which indicates an increased amount of α-helical structures of the tetramer in 10 mM phosphates.

When histone (H3 + H4) tetramer-DNA complexes were prepared in phosphate buffer and subsequently dialyzed to EDTA buffer, the thermal denaturation profiles are very much different from those of histone (H3 + H4)-DNA complexes directly mixed in EDTA buffer although the CD spectra of these two complexes are nearly identical. The melting profiles of histone (H3 + H4) tetramer-DNA complexes have an extra melting band at around 65°. These melting profiles resemble those of native chromatin. Another similarity between the tetramer-DNA complexes and the native chromatin is in their β value which is 2.8 in the former and 3.0 - 3.5

in the latter. Compared to a β value of 5.0 to 6.7 for histone H3-DNA, H4-DNA and (H3 + H4)-DNA complexes prepared by direct mixing in EDTA buffer, the tetramer-DNA complex is closer to the native chromatin.

DNA complexes of histone (H3 + H4) tetramer and histone (H3 dimer + H4) tetramer have nearly identical thermal denaturation and CD properties. Complexes formed between DNA and histone (H3 + H4) tetramer or histone (H3 dimer + H4) tetramer also prevent the precipitation of the complexes when histone H3 or H4 was used alone. These results suggest the existence of a subunit of histone (H3 + H4) tetramer, using a histone H4 dimer and a H3 dimer with or without disulfide bond. The tetramer then interacts with DNA in a manner similar to that in chromatin. Since a parallel dimer of histone H4 has been shown to exist (Li et al., 1972) and a parallel dimer of histone H3 is implicated by the formation of disulfide bond in the C-terminal region of this molecule, it is possible that the tetramer is formed by hydrophobic contact of the H3 and H4 dimers and then interacts with the DNA. In fact, these results are one of the bases used for a model of chromatin structure recently proposed (Li, 1975).

CHAPTER VII

CONCLUDING DISCUSSION

Recent revised primary sequence shows that calf thymus histone H3 is heterogeneous with respect to its Cys content (Patthy and Smith, 1975). Eighty percent of the molecules contain two Cys residues and another 20% contain one Cys residue. This is in agreement with an earlier report (Marzluff et al., 1972), which showed that calf thymus histone H3 monomer isolated by their procedure contains a minor fraction with an unoxidized Cys and a major fraction with two Cys forming an intramolecular disulfide bond. The two forms of histone H3 were seperated by gel filtration after oxidizing the histone H3 containing a single Cys in guanidine-HCl into histone H3 dimer via intermolecular disulfide linkage. Marzluff et al.,(1972), however, were unable to find oligomeric histone H3 which was reported by Panyim et al. (1971).

The oxidation results in Chapter IV (Figures 20,21, and 22) can be used to explain this discrepancy. Oxidation in the presence of a denaturating agent, such as guanidine-HCl or urea, results in the convertion of only a minor fraction of histone H3 into the dimeric form (Figure 20).

This can be interpreted as the convertion of those histone H3 molecules containing two Cys residues into cyclic monomer with intramolecular disulfide bond and the convertion of the remaining histone H3 molecules containing one Cys residue into the dimeric form. Oxidation in the absence of denaturating agent, in particular, in slightly alkaline conditions (0.1M Tris-HCl, pH 8.0), leads to the formation of dimeric and oligomeric forms (Figures 21 and 22). This is probably due to the formation of more secondary structures in histone H3 in 0.1M Tris-HCl, pH 8.0, as shown by CD (Figure 25, Chapter IV), which prohibit the formation of intramolecular disulfide bond, possibly through steric hindrance.

Histone H3 monomer-, dimer-, and oligomer-DNA complexes prepared by the method of direct mixing in EDTA buffer yield nearly identical thermal denaturation curves with two well-defined melting bands and a β value around 6.5 amino acid per nucleotide. These results indicate that, although there are variations in the oxidation state at the Cys residues in the C-terminal region, these variations have no substantial effects on their binding property to DNA. In other word, it is highly probable that only the more basic regions of these molecules bind to DNA and that the C-terminal (hydrophobic) region is not firmly bound to DNA and is free to interact with other histones.

Two polypeptide-bound DNA melting bands (T'_{mI} and T'_{mII}) were obsereved when poly-Arg and poly(Arg^{87},Orn^{13}) were used for complexes prepared by the direct mixing method. However, when protamine, a protein containing 67% Arg, was used for complex formation with DNA only one protein-bound DNA melting band was found. It is not surprising then that the complexes of Arg-rich histones (histone H3 contains 13.3% Arg and H4 contains 12.7%

Arg residues) and DNA prepared by direct mixing method showed only one T_m' protein-bound melting band (T_m' at 90°C) corresponding to the T_{mI}' of the poly-Arg-DNA complexes and the T_m' of protamine-DNA complexes.

For poly-Arg, poly(Arg^{87},Orn^{13})-, and protamine-DNA complexes, there are respectively, 0.72, 1.05, and 1.38 amino acids per nucleotide in the peptide bound regions. These correspond respectively to 0.72, 1.05, and 0.92 basic residues (Arg) per nucleotide in the bound regions and indicate that for poly(Arg^{87},Orn^{13})- and protamine-DNA complexes, the positive charges of the polypeptides nearly neutralized the negative charges of the DNA backbone. Histone H3 and H4-DNA complexes prepared by the direct mixing method have a β value in the bound regions of 6.7 and 5.2 respectively. These correspond to 1.5 and 1.3 basic residues per nucleotide in the bound regions. However, since both histone H3 and H4 contain not only basic residues (Lys + Arg) but also acidic residues (Glu + Asp), the apparent number of basic residues per molecule can be calculated as the number of basic residues minus the number of acidic residues. The number of apparent basic residues per nucleotide in the bound regions for histone H3-DNA complexes is 0.99 and for H4-DNA complexes, 0.92. These results indicate that the phosphate groups in the bound regions are nearly neutralized by basic residues in histone and that the acidic residues within the histone molecules are also neutralized by the basic residues.

The CD spectra ($\Delta\mathcal{E}_b^D$) of DNA base pairs bound by protamine (Fig 17) at the CD band near 275 nm is very similar to the CD spectra of DNA base pairs bound by histone H3 or H4 (Figs. 33a and 46a). The amplitude of the CD band is about 2/3 that of free DNA. There is also a slight red shift for the CD band near 275 nm for all the three complexes. These results are

in contrast to the much more severe red shift and amplitude reduction found for the complexes prepared with poly-Arg or poly(Arg^{87},Orn^{13}) (Figs. 15 and 16). The phenomenon of red shift and amplitude reduction for the CD band near 275 nm are indicative of a DNA conformation between B and C forms for the protein-bound base pairs. Similar CD results have been obtained for DNA in high NaCl concentration (Tunis and Hearst, 1968; Tunis-Schneider and Maestre, 1970; Li et al., 1971). The binding of NaCl to DNA resulting in the transformation of DNA from B to C form in high salt have been regarded as the effect of both charge neutralization and dehydration of the whole DNA molecule (Tunis-Schneider and Maestre, 1970). Basic proteins binding to DNA should also leads to both neutralization of DNA charges and the dehydration of the DNA molecule. The dehydration of DNA could be due to the exclusion of water ,olecules from the vicinity of the DNA by the hydrophobic side chains present in the basic proteins. The methylene groups of Lys and Arg side chains should also exclude water. Poly-Arg and poly(Arg^{87},Orn^{13}) binding lead to a CD spectra approximately equals to the effect of 6 M NaCl, while protamine and histone H3 and H4 binding lead to one that is similar to the effect of a lower concentration of NaCl.

The CD spectra of the model systems differ greatly from those of histone H3 and H4-DNA complexes in the regions of 235 and 220 nm. The poly-Arg-DNA, poly(Arg^{87},Orn^{13})-DNA, and protaime-DNA complexes showed no negative CD band near 220 or 235 nm. In contrast, large negative CD bands near 220 and 235 nm are found for the histone H3- or H4-DNA complexes prepared by direct mixing method corresponding to the secondary structures of the bound histones. In this respect, the CD spectra of histone H3-DNA

complexes prepared by the gradient dialysis with urea method is very similar to poly-Arg-DNA and poly(Arg^{87},Orn^{13})-DNA complexes in that there are large changes in the CD band near 275 nm and no significant negative CD band near 235 nm. This would indicate that histone H3, in complexes prepared by gradient dialysis with urea, contained similar secondary structures in the bound state as with poly-Arg and poly(Arg^{87},Orn^{13}).

There are two possible explanations for this phenomenon of low CD spectra at 220 and 235 nm. Firstly, perphaps the binding of poly-Arg, poly(Arg^{87},Orn^{13}) and protamine to DNA as well as the binding of histone H3 and H4 to DNA by the gradient dialysis with urea method lead to a blue shift of the $n \rightarrow \pi^*$ transition of the amide groups so that their CD contribution near 220 and 235 nm become greatly reduced. Another possibility for the reduction of CD spectra is that the amide groups in the peptide-DNA complexes have a conformation which is none of the three conformations commonly found for proteins in solution, i.e., α helix, β sheet, and random coil. This type of conformation may be a results of tight binding between basic polypeptides or proteins with DNA.

The melting curves of histone H3-DNA and histone H4-DNA complexes prepared by gradient dialysis with or without urea are usually not so well defined as they are for directly-mixed complexes. CD spectra of histone H3-DNA and H4-DNA complexes prepared by these methods show a strong red-shift in the DNA CD band near 275 nm. However, an equimolar mixture of both histone H3 and histone H4 yield complexes that

have better defined melting curves. The DNA CD band near 275 nm is also less red-shifted and has a smaller reduction in amplitude. The presence of both histones during complex formation also reduces the tendency of precipitation which has been observed when individual histone was used. All these results imply that histone H3-histone H4 interaction could possibly exist in NaCl with or without urea and that the histone H3-histone H4 subunit could form a better complex with DNA.

Thermal denaturation and CD studies of Arg-rich histone-DNA complexes indicate that preformation of histone (H3 + H4) tetramer before complexing yields a complex that is most similar to thermal denaturation and CD properties of native chromatin. Native chromatin has two histone-bound DNA melting bands at 71° and 82°. Histone (H3 + H4) tetramer-DNA complex has melting bands at 65° and 90° for the protein-bound base pairs. Their are 3.0-3.5 amino acid residues per nucleotide in the histone-bound regions in native chromatin. Approximately 2.8 amino acid residues per nucleotide are found in histone (H3 + H4) tetramer bound regions. The CD band near 275 nm for histone (H3 + H4) tetramer-DNA complexes is reduced in amplitude and slightly red-shifted as is the case in chromatin. Similarly, prominent negative CD bands near 220 nm are found for both the chromatin and histone (H3 + H4) tetramer-DNA complexes. Although these results do not conclusively prove that the tetramer of histone (H3 + H4) under present experimental condition may be identical to what might exist in native chromatin, the ability to reproduce thermal denaturation and CD properties of chromatin in these complexes do suggest that (H3 + H4) tetramer could probably be a native subunit in chromatin.

Using histone H3 dimer and histone H4, the complexes with DNA show melting and CD properties very similar to those when histone H3 monomer

and histone H4 were used. These results imply that, perhaps, a parallel histone H3 dimer with or without disulfide bond is a fundamental subunit in the tetramer. Since a parallel histone H4 dimer does exist in solution (Li et al., 1972; Li, 1973), it seems likely that two parallel dimers of H3 and H4 form a bigger subunit of tetramer, possibly through hydrophobic interaction in the C-terminal regions as recently proposed inaa model of chromatin structure (Li, 1975).

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